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**THE MICROBIAL OXIDATION OF  
METHANESULFONIC ACID IN THE MARINE  
ENVIRONMENT.**

by

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### **Declaration**

The work contained in this thesis was the result of original research carried out by myself under the supervision of Dr. J.C. Murrell (University of Warwick) and Prof. N.J.P. Owens (University of Newcastle-upon-Tyne). All the sources of information have been specifically acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

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### Abstract

The biogeochemical transformations related to methanesulfonic acid (MSA) formation and degradation are discussed, with reference to the role of marine bacteria and the phylogeny and biochemistry of methylotrophic bacteria are briefly reviewed. The aims of the work presented were [i] to isolate novel MSA utilising bacteria from both seawater and freshwater samples, [ii] to characterise these isolates and [iii] to elucidate the mechanisms by which MSA is metabolised in these isolates.

Isolation procedures for the enrichment of MSA-oxidizing bacteria, from a wide range of seawater and freshwater sites, are described. Four methylotrophic bacterial strains, TR3, PSCH4 (marine isolates), FW2 and FW6 (freshwater isolates), capable of growth on MSA as a sole carbon source were isolated from the environment. MSA metabolism in strains TR3 and PSCH4 was initiated by an inducible NADH-dependant mono-oxygenase, which cleaved MSA into formaldehyde and sulfite. Formaldehyde was assimilated via the serine pathway. Cell suspensions of bacteria grown on MSA completely oxidized MSA to carbon dioxide and sulfite with a MSA: Oxygen stoichiometry of 1.0: 2.0. Oxygen electrode-substrate studies indicated the dissimilation of formaldehyde to formate and CO<sub>2</sub> for energy generation. Methanol was not an intermediate in MSA metabolism, although the strains could grow on methanol and other one-carbon compounds, as well as a variety of heterotrophic substrates. Initial studies of strains FW2 and FW6 indicated that they probably metabolised MSA in a similar way to the marine strains. Carbon dioxide was not fixed by ribulose biphosphate carboxylase in strains TR3 and PSCH4.

These novel facultative methylotrophs have the ability to mineralize MSA and may play an important role in the cycling of global sulfur, since MSA can be a major product from the oxidation of DMS, the principal biogeochemical organosulfur gas in the environment.

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# Abbreviations used in the text.

AMS.....	Ammonium mineral salts medium
ANMS.....	Ammonium nitrate mineral salts
ATP.....	Adenosine triphosphate
BSA.....	Bovine serum albumin
C <sub>1</sub> .....	Carbon compound without a carbon to carbon bond (e.g. methane, dimethyl-sulfide)
C <sub>3</sub> .....	3 carbon compounds
CCN.....	cloud condensation nuclei
CH <sub>4</sub> .....	methane
CLAW.....	Charlson Lovelock Andreae Warren (feedback theory)
cpm.....	counts per minute
CTD.....	Conductivity, Temperature, Depth (shipboard sampling device)
D.....	dilution rate
d.....	days
Da.....	Daltons
DCPIP.....	dichloroindophenol
DMS.....	dimethyl sulfide
DMSO.....	dimethyl sulfoxide
DMSP.....	dimethyl sulfoniopropionate
DNA.....	deoxyribonucleic acid
ε.....	extinction coefficient
ESA.....	ethanesulfonate
F.....	flow rate
FDH.....	formate dehydrogenase
FID.....	flame ionisation detector



FRG.....	Germany
FW.....	freshwater
g.....	gravity (centrifugal force)
GC.....	gas chromatograph
HEPES.....	N-[2-hydroxyethyl] piperazine- N'-[2-ethanesulfonic acid]
HPR.....	hydroxypyruvate reductase
HPS.....	hexulose phosphate synthase
h.....	hours
IC.....	ion chromatograph
icl.....	isocitrate lyase
KDPG.....	2-keto, 3-deoxy, 6-phospho-gluconate
K <sub>m</sub> .....	Michaelis constant
l.....	litres
LAS.....	linear alkylbenzene sulfonate
M.....	molar
m.....	meters
MA.....	Mid-Atlantic
MAMS.....	marine ammonium mineral salts medium
MDH.....	methanol dehydrogenase
min.....	minutes
MMA.....	monomethylamine
MMPA.....	3-methiopropionate
Mol.....	moles
Mol%G+C.....	percentage content of guanine and cytosine of DNA
MPA.....	3-mercaptopropionate
MS.....	nitrogen-free mineral salts medium
MSA.....	methanesulfonic acid

MT.....	methanethiol
n.....	number of carbons (e.g. as in $n = 2$ )
n/r.....	not reported
n/t.....	not tested
NA.....	Nutrient agar
NAD <sup>+</sup> .....	nicotinamide adenine dinucleotide
NADH.....	reduced nicotinamide adenine dinucleotide
NADP <sup>+</sup> .....	nicotinamide adenine dinucleotide phosphate
NADPH.....	reduced Nicotinamide adenine dinucleotide phosphate
NMS.....	nitrate mineral salts medium
NO <sub>x</sub> .....	nitrogen oxides (non-specific)
NS.....	North Sea
OD <sub>x</sub> .....	optical density of a solution at x nm
PAGE.....	polyacrylamide gel electrophoresis
PCR.....	Polymerase Chain Reaction
PES.....	phenazine ethosulfate
PMS.....	phenazine methosulfate
PQQ.....	pyrrolo-quinoline quinone
PS.....	Plymouth Sound
R.....	non-specific organic group (e.g. methyl)
RNA.....	ribonucleic acid
RNase.....	ribonuclease
rpm.....	revolutions per minute
RuBISCO.....	ribulose-1,5-bisphosphate carboxylase
RuBP.....	ribulose bisphosphate
RuMP.....	ribulose monophosphate
s.....	seconds

SDS.....	sodium dodecyl sulfate
SGA.....	serine glyoxylate aminotransferase
SL.....	South Lynn
TB.....	Thorpe Bay
TE.....	Tris-EDTA buffer
TR.....	Tamar River Estuary
V.....	volts
v/v.....	volume per volume
$V_{\max}$ .....	maximum velocity
w/v.....	weight per volume
WS.....	Wakering Steps
$\mu_{\max}$ , $\mu_m$ .....	maximum specific growth rate

**THE MICROBIAL OXIDATION OF METHANESULFONIC ACID**  
**IN THE MARINE ENVIRONMENT**

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# **THE MICROBIAL OXIDATION OF METHANESULFONIC ACID IN THE MARINE ENVIRONMENT.**

## **CHAPTER 1: BACKGROUND**

In this introductory chapter to the thesis being presented, I shall review the occurrence of MSA, the processes associated with the formation of MSA, sulfur-specific biodegradative pathways of relevance to MSA metabolism, the phylogeny and biochemistry of methylotrophy and particular topics in marine microbial ecology of direct relevance to the physiology and ecology of bacteria likely to utilise MSA.

### **1.1. METHANESULFONIC ACID AND THE MARINE ORGANIC SULFUR CYCLE.**

Few studies have concerned the biological cycling of methanesulfonic acid (MSA), but many have concentrated on the production of MSA in the natural environment, including field measurements, reaction mechanisms and the likely effects of MSA on climate changes. In order to study the biological cycling of MSA, it is necessary to consider the role and importance of MSA in the marine environment, and the interactions of MSA with the environment. By considering these factors,

successful targeted enrichment programmes can be developed with regard to the environments where MSA will be found.

#### **1.1.1. Occurrence of methanesulfonic acid.**

Methanesulfonic acid ( $\text{CH}_3\text{SO}_3\text{H}$ ) is a colourless strong acid. It is produced industrially as the free acid, but may be obtained as the naturally occurring sodium salt. Industrially, MSA is used as a non-aqueous solvent, particularly in the production of high performance polymers (Roitman et al., 1994). It is highly hygroscopic; absorption of water can have a marked effect on the physical properties of MSA (Roitman et al., 1994). This acid is unusually stable, and is resistant to chemical oxidation/hydrolysis (e.g. boiling alkali hydrolysis).

In addition, MSA is a major component of the marine organic sulphur cycle. A range of environmental concentrations is shown in Table 1.1, showing geographical variations. These quantities (including ice core data) reflect atmospheric concentrations at the time of deposition. MSA has been detected in marine precipitation (Charlson et al., 1987), but the total amount of MSA partitioned into sea water, by wet or dry deposition, is largely unknown. This is primarily due to analytical problems, described briefly in section 1.1.4.

LOCATION	[MSA] RANGE	SAMPLE	REF.
Antarctica	0-0.84 nmol m <sup>-3</sup>	Aerosol	Savoie et al., 1992
Antarctica	0-1.51 $\mu$ mol l <sup>-1</sup>	Snow	Udisti et al., 1991
Plymouth Sound	0-5.0 nmol m <sup>-3</sup>	Aerosol	Watts et al., 1987
Pacific Ocean	0-0.80 nmol m <sup>-3</sup>	Aerosol	Bates et al., 1992
Sargasso Sea	0-0.81 nmol m <sup>-3</sup>	Aerosol	Kolaitis et al., 1989
Miami, Fl.	0-1.6 $\mu$ mol l <sup>-1</sup>	Rainfall	cited Udisti et al., 1991

**Table 1.1. Typical determinations of MSA concentration from a variety of sources.** Differing units used reflects standard units used in aerosol and liquid (i.e. rainfall) determinations of MSA concentration.

MSA is highly soluble and probably partitions into seawater extremely efficiently (Clegg and Brimblecombe, 1985). The partition coefficient of MSA has been estimated at between 0.9 to 1.0 (Chatfield and Crutzen, 1990). In the case of snow and ice samples, it would be reasonable to assume that the concentration of MSA would be similar in melt waters.

#### 1.1.2. The formation of MSA from natural sources.

MSA is a major photochemical oxidation product of dimethylsulfide (DMS) (Grosjean, 1984), which is derived principally from dimethylsulfoniopropionate (DMSP). Challenger and Simpson (1948) first determined that DMSP was a precursor of DMS, in the marine red alga *Polysiphonia lanosa*.



DMSP concentrations in sea water are divided into soluble DMSP and particulate DMSP. The concentrations of these are typically up to 6 nmol l<sup>-1</sup> and 15 nmol l<sup>-1</sup> respectively (Andreae, 1990). The average intracellular concentrations of DMSP are 0.2-0.4 mol DMSP l<sup>-1</sup> cell volume (Andreae, 1990).

The function of DMSP is uncertain, but it has been suggested that DMSP has a osmostatic, osmoregulatory (Vairavamurthy et al., 1985) and cryoprotectant function (Kirst et al., 1991) in a number of species of marine phytoplankton (Turner et al., 1988, Belviso et al., 1990), algae (Kirst et al., 1991) and salt marsh grasses (Dacey et al., 1987). A summary table (Table 1.2.) shows a variety of genera that produce DMSP.

Some DMSP is abiotically oxidized in seawater to DMS at a slow rate (Andreae, 1990), however most DMSP is biologically oxidized. Biologically produced DMS is derived primarily from the action of DMSP lyases. Both bacterial and algal DMSP lyases have been identified (Stefels and van Boekel, 1993, Visscher et al., 1992, Ledyard and Dacey, 1994, De Souza and Yoch, 1995). DMSP is enzymatically cleaved to DMS and acrylic acid within the cell (Stefels and van Boekel, 1993) for physiological reasons, as yet, unknown, or by bacterial consumption (Visscher et al., 1992), probably upon cell death. The lyases responsible for this seem to be, in at least

some cases, membrane bound (Cantoni and Anderson, 1956). DMSP may also be demethylated via an alternative bacterial pathway to 3-methiolpropionate (Kiene, 1993). The relative inputs of these two pathways is uncertain, largely due to insufficient knowledge of the demethylation pathway.

GROUP	EXAMPLE	REF.
Macroalgae	<i>Polysiphonia lanosa</i>	Challenger and Simpson (1948)
Dinoflagellates	(some species)	Andreae (1990)
Coccolithophores	<i>Hymenomonas carterae</i>	Vairavamurthy et al., (1985)
Chrysophytes	(some species)	Andreae (1990)
Prymnesiophyte	<i>Phaeocystis</i> sp.	Steffels and van Boekel (1993)
Cyanobacteria	<i>Microcoleus chthonoplastes</i>	Visscher and van Gernerden (1991)
Salt Marsh Grasses	<i>Spartina alterniflora</i>	Dacey et al., (1987)

**Table 1.2. DMSP producers in the marine environment.**

DMS can also be derived from a number of different compounds in various habitats. Methoxylated aromatics, such as 3,4,5-trimethoxybenzoate, have been identified as precursors of DMS in anaerobic sediments with the conversion being carried out by *Pelobacter* spp.

(strict anaerobes) (Bak et al., 1992).

Dimethylsulfoxide (DMSO) and dimethyldisulfide (DMDS) are also metabolised to DMS in anoxic salt marsh sediments (Kiene and Capone, 1988) by undefined anaerobic bacteria.

DMS is a volatile liquid, so a significant proportion is lost to the atmosphere. The quantities produced and lost varies according to latitude (Bates et al., 1992). High concentrations of DMS are generally observed above productive areas, such as the North sea, whereas above open oceans concentrations are lower. Andreae (1990) estimates that the total annual emissions from the oceans is 0.6 to 1.6 Tmol S yr.<sup>-1</sup>, with typical surface water (0-50m, NW Atlantic Ocean) concentrations estimated at 4-7 nmol l<sup>-1</sup>.

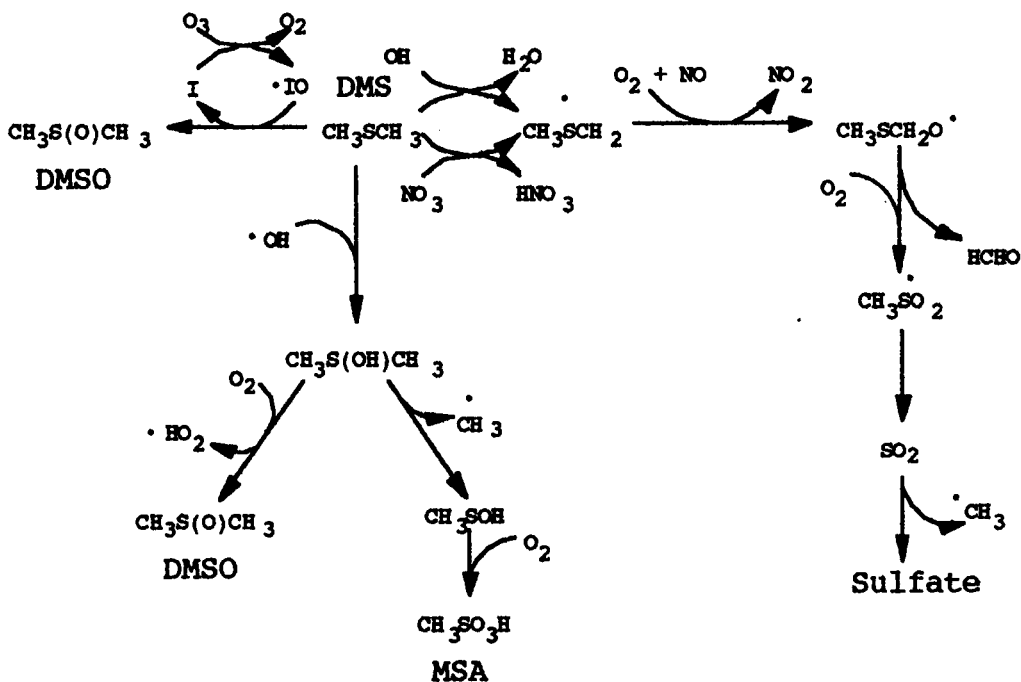
Once DMS enters the atmosphere, it is rapidly oxidised to a number of products; sulphur dioxide, non-sea salt sulfate, and MSA. The relative proportions of these depends on local conditions, such as free radical availability in the atmosphere. For instance, MSA is the dominant product over the Southern and Antarctic Oceans, whereas in other regions, it may be sulfur dioxide (Andreae, 1990). Mihalopoulos et al. (1992) suggests that MSA accounts for 20-25% of oxidised DMS, on a molar basis. A summary of these processes is shown in Figure 1.1.

**Figure 1.1. Main features of the organic marine sulfur cycle.**

OH· and NO<sub>x</sub> radicals are required to oxidize DMS to MSA and sulfur dioxide in the atmosphere (Jensen et al., 1992, Platt et al., 1990, Grosjean, 1984). OH· radicals carry out the day-time reaction, and NO<sub>x</sub> the night time mechanism. Little is known about these mechanisms and there is much conflicting evidence. Only the OH· route has been studied in detail at the mechanistic level (Grosjean, 1984).

The series of reactions for the oxidation of DMS are complex. Two pathways are present; addition (which eventually produces MSA), and abstraction (producing sulfur dioxide). The abstraction pathway can also yield MSA under certain conditions, which requires

$\text{NO}_x$ . It is not clear which pathway predominates. Local conditions may play a role (i.e. geographical position, presence of industrial activity), since the addition pathway has been shown to be temperature sensitive. This is thought to explain increased MSA concentrations at locations of low temperature, e.g. Antarctica. The relevant reactions are summarised in Fig. 1.2.



**Figure 1.2. Reaction schemes showing the atmospheric oxidation of DMS (after Andreae, 1990 and Jensen et al., 1992).**

It has been suggested that  $\text{NO}_x$  mediated MSA oxidation routes favour the sulfur dioxide mechanism (Andreae, 1986), thereby favouring this mechanism in coastal urban (i.e. polluted) areas. However, recent data suggest otherwise. Mihalopoulos et al. (1992) state that in  $\text{NO}_x$  containing systems (in chamber

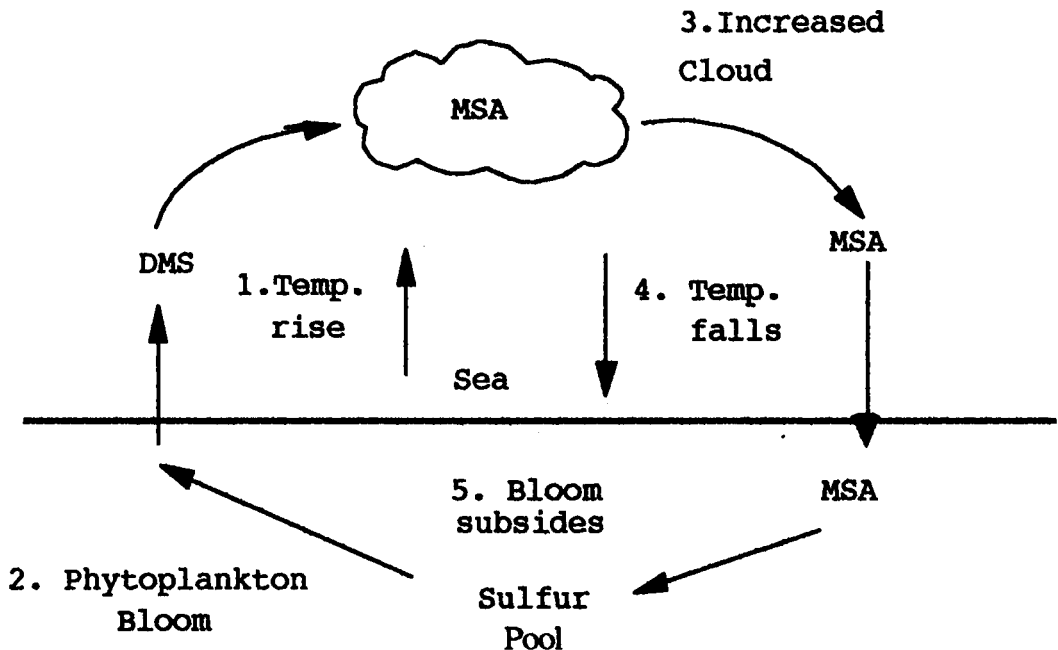
experiments), SO<sub>2</sub> and MSA yields were 30% and 50-70% respectively. In NO<sub>x</sub> free systems, the respective yields were 70-90% and less than 1% respectively. The authors suggest that crossreactivity of radicals produced by the oxidation processes complicates the picture further. One could conclude that MSA production is more sensitive to the presence of pollutant generated radicals than SO<sub>2</sub>. Indeed, diurnal measurements suggested that the night-time NO<sub>x</sub> route of MSA production is the most important (Milhalopoulos *et al.*, 1992).

The subsequent fate of MSA is largely unknown. Once in the atmosphere, MSA rapidly becomes bound to salt particles, or goes on to become cloud condensation nuclei (CCN). The salt bound MSA is probably transported to land or sea water by dry/wet deposition (Andreae, 1986). As previously inferred, one would assume that the bulk of the MSA is deposited at the point of production. Field measurements (Mihalopoulos, 1992) suggest that this process would be complete in a matter of hours. Ice core measurements (Saigne and Legrand, 1987) have shown that MSA has not been accumulating. Since it is therefore being cycled, the assumption is that it is being cycled to sulfate.

#### **1.1.3. The possible role of MSA in climate models.**

MSA has an important role as a source of cloud condensation nuclei (CCN). CCN are the particles

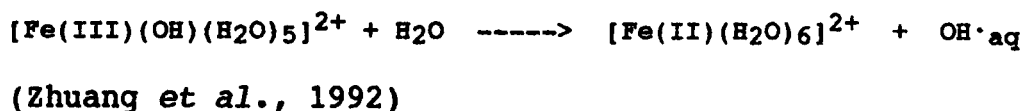
responsible for cloud albedo, which is responsible for ultra violet reflection. This induces cooling of air temperatures. Charlson et al. (1987) have proposed a feed-back mechanism (known as the CLAW hypothesis), illustrated in Fig. 1.3.



**Figure 1.3. The possible interactions of the sulfur cycle and climate (after Charlson et al., 1987).**

The theory states that as global temperatures rise, so surface sea temperatures will rise. This will produce thermoclines within the water column, and so induce phytoplankton blooming. More DMSP is produced, hence more DMS is formed. Conversion of DMS in the atmosphere to MSA will yield more CCN particles, so inducing a period of cooling, where upon sea temperatures drop, and blooming subsides.

This theory could be further developed to encompass close coupling of the iron and sulfur cycles. Zhuang et al. (1992) have suggested that the cycling of iron (II) and the marine sulfur cycle are closely coupled, in that iron plays a limiting role in both the biological and atmospheric cycles. Iron is now accepted as the limiting nutrient in remote areas, and so will control production of DMS. Iron (II) atmospheric chemistry produces OH radicals via the following scheme:



Martin et al. (1990) suggested that seeding the oceans with iron (II) would stimulate primary production to reduce atmospheric carbon dioxide concentrations. The above reaction scheme would suggest that cloud formation would be also stimulated, by production of hydroxyl radicals. This may stimulate daytime oxidation of DMS to MSA. If the CLAW hypothesis is correct, global cooling would occur. This CLAW hypothesis has not been verified. Ice core studies have produced conflicting evidence, regarding MSA concentrations during the last glacial period. Saigne et al. (1987) report that MSA concentrations increased during the last ice age. However, Hansson and Saltzman (1993) report decreasing MSA concentration during an ice age. In addition, only certain species of



phytoplankton produce DMSP and DMS. Whether the theory can ever be verified remains to be seen.

#### 1.1.4. Measurement of MSA in the environment.

Several methods to measure MSA are available, including Dionex ion-chromatography (Watts, 1989, Saigne et al., 1987), gas chromatography and laser microprobe mass analysis (LAMMA) (Kolaitis et al., 1989). Of these, Dionex chromatography is considered the most suitable. LAMMA is expensive, and not suitable for *in-situ* measurements, and gas chromatography is unreliable (Watts et al., 1987). A method has been developed to determine concentrations in sea water using the Dionex system. Essentially, the protocol involves pretreatment and filtration of the sample with silver salts, to remove halides that interfere with the signal for MSA, followed by 1000-fold concentration of the sample. The method is laborious and expensive, making it unsuitable for extensive field studies in its present form (S. Watts, personal communication, see Appendix II). An estimate of surface sea water concentrations can be made, based on existing atmospheric data, and by making certain assumptions about the behaviour of MSA salt. For the area around the British Isles, an estimate of 0.1 ppb can be made. Measurements of the Bristol Channel area (by Dionex chromatography) show concentrations to be several orders of magnitude higher (S. Watts, personal communication.).

## **1.2. OVERVIEW OF SULFUR BIOCHEMISTRY.**

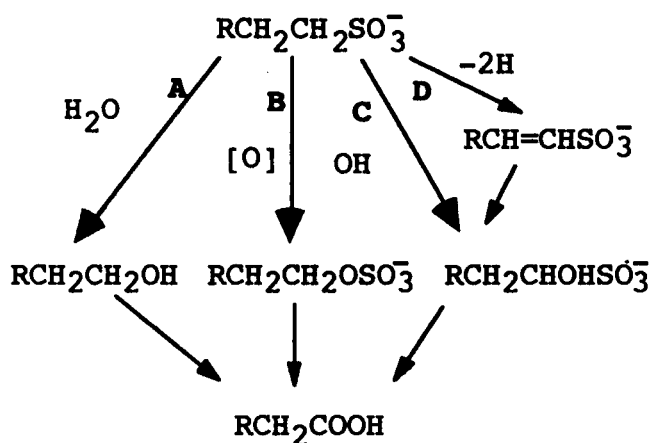
Organic sulfur compounds are broken down both anaerobically and aerobically in prokaryotic and eukaryotic systems. For this discussion I shall be concentrating on sulfur compounds of most relevance when considering the biodegradation of MSA in the marine environment; namely organic sulfonates and organic sulfide compounds (and related substrates). The justification for this is that [a] aerobic metabolism may follow patterns of degradation found in other organic sulfonates and [b] marine bacteria capable of utilizing MSA may be capable of utilising other related sulfur compounds, such as DMS, DMSP and methanethiol.

### **1.2.1. Biodegradation of alkyl and aryl sulfonates.**

The biodegradation of both alkyl and aryl sulfonates has been studied in some detail over the last 30 years. This has been mainly from an industrial point of view, particularly with regard to the biodegradability of linear alkylbenzene sulfonates (LAS), used extensively as industrial surfactants (Kelly and Smith, 1990).

The biodegradation of LAS and n-alkane sulfonates by anaerobic and aerobic bacteria (Chien et al., 1995, Seitz et al., 1993, Lee and Clark, 1993, Locher et

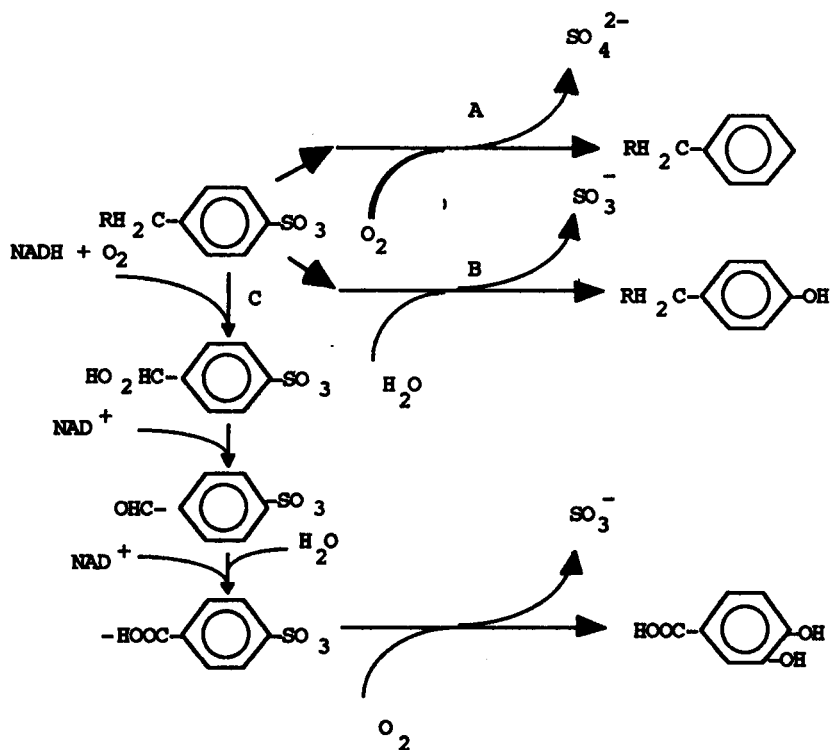
al., 1989, Willets and Cain, 1972, Thyse and Wanders, 1974), yeasts (Uria-Nickelsen et al., 1993b, Willets, 1973) and algae (Biedlingmaier and Schmidt, 1983) have been extensively documented in the literature. Thyse and Wanders (1974) proposed a series of theoretical reaction schemes for the breakdown of n-alkane sulfonates (Fig. 1.4.) by *Pseudomonas* spp.



**Figure 1.4. Possible sulfonate biodegradative pathways (after Thyse and Wanders, 1974).** A; Hydrolysis of the carbon sulfur bond. B;  $\alpha,\beta$ -Dehydrogenation. C; Insertion of oxygen into the carbon-sulfur bond. D;  $\alpha$ -Hydroxylation (oxidation).

$\alpha$ -Hydroxylation was found to occur within the strains *Pseudomonas* AJ 1 and *Ps.* AJ 2. An NADH-dependant mono-oxygenase was proposed for the initial hydroxylation. The unstable bisulfite-aldehyde adduct produced spontaneously broke down to form the carboxylic acid and sulfite.

The metabolism of aryl sulfonates proceed via pathways where the initial or early steps are the desulfonation of the substrate, rather than modification elsewhere of the molecule (e.g. cleavage of the benzene ring). Cain (1971) reported the oxidation route of benzene sulfonates by *Bacillus C1*. It was reported that the sulfite group was replaced directly with a hydroxyl group by a "desulfonating enzyme". This enzyme was repressed by sulfite accumulating in the medium (Fig. 1.5). Willets reported a similarly named enzyme in the fungus, *Cladosporium resinae* (Willets, 1973). Its mode of action was entirely different however. The alkyl side group of the aryl sulfonate was carboxylated, with subsequent release of the sulfite moiety as sulfate (Fig. 1.5.). Locher et al. (1989) reported a different route for the degradation of toluene sulfonate by *Pseudomonas testosteroni*. Desulfonation did not occur initially. The methyl side group was initially oxidized, giving sulfobenzoate, which was further oxidized to protocatechuate with the simultaneous release of sulfite. This is in contrast to the other routes discussed previously where the desulfonation is the initial step (Fig. 1.5).



**Figure 1.5. Biodegradation of arylsulfonates in fungi (A, Willets, 1973), *Bacillus* sp. (B, Cain, 1971) and *Pseudomonas* sp. (C, Locher et al., 1989. N.B. In this scheme, "R" = OH).**

Other studies have considered organic sulfonates as potential sulfur sources for a variety of bacteria. Mostly, this has been done with enteric organisms (Uria-Nickelsen et al., 1993a, 1994) and *Pseudomonas* sp. (Seitz et al., 1993). In all the organisms screened, the sulfonate tested (MSA, dodecanesulfonate, HEPES, taurocholate, cysteate, isethionate, taurine) could not act as sole carbon and energy source, only as a sulfur source. The sulfonate moiety appears to be directly cleaved from the main molecule, giving rise to sulfite in the cytoplasm. It is surprising that growth was not supported with these sulfonates as sole carbon and energy source as, in the

authors' view, the residue was probably hydroxylated to give alcohols that should have been easily assimilated, at least in the *Pseudomonas* spp. described. It was suggested that sulfite or sulfate may be inhibitory to further metabolism of these alcohol residues. Sulfate also appeared to compete with sulfonate uptake. Similarly, Biedlingmaier and Schmidt (1983) also reported the ability of *Chlorella fusca* to utilise sulfonates as sulfur sources, with the concomitant release of sulfite. Recently, Chien et al. (1995) showed the assimilation of alkyl and aryl sulfonates under anaerobic conditions by *Clostridium pasteurianum*. *C. pasteurianum* was shown to assimilate taurine, isethionate and toluenesulfonate. MSA was not assimilated. The assimilation pathway has not been determined for this organism.

There have been few studies looking at the catabolism of MSA. Uria-Nickelsen et al. (1993a) showed that certain strains of enteric bacteria can utilise MSA as a sulfur source. However, given that their findings indicate that sulfate is preferentially used to sulfonates, it is likely that this system would be minor pathway in seawater (where sulfate concentrations are in excess). Baker et al. (1991) isolated a methylotrophic soil bacterium, strain M2, able to utilise MSA as a source of carbon, and producing sulfite and formaldehyde concurrent with that derived from MSA.

It is well known that taurine (2-aminoethanesulfonic acid) serves as a sulfur source for *E. coli* (Kondo et al., 1971, Kondo and Ishimoto, 1972), via a well characterised pathway. In the context of the marine environment, this is a significant sulfonate. It is found specifically in fish eurythrocytes and euryhaline crabs and in mammalian cells it may serve as an osmoregulator (McLaggan and Epstein, 1991). It is likely, therefore, to be found in significant quantities in seawater. Bacteria that utilise MSA as a carbon source may also be able to utilise taurine and related compounds.

#### **1.2.2. Biodegradation of DMS.**

A discussion of the biodegradation of dimethylsulfide (DMS) would be relevant here since not only is it a direct precursor of MSA, but also, since it is a C<sub>1</sub> compound, a potential methylotrophic growth substrate, and hence of use in developing a targeted enrichment programme.

Bacterial DMS degradation has been demonstrated in both aerobic and anaerobic marine environments (Kiene, 1993). DMS degradation has been measured in both sediments (aerobic and anoxic) and in the water column (Kiene, 1993). The two principal compounds arising from biodegradation of DMS are methanethiol (MeSH) and dimethylsulfoxide (DMSO). It has also been suggested that methanethiol and dimethyldisulfone (DMSO<sub>2</sub>) are

potential precursors of MSA (Kiene, 1993, Visscher et al., 1995).

Aerobic bacteria such as *Thiobacillus thioparus* (Kelly and Smith, 1990) and methylotrophic bacteria (e.g. *Hyphomicrobium* S.) (De Bont et al., 1981) have been shown to utilise DMS as a carbon and energy source. In the case of the hyphomicrobia, De Bont et al. (1981) showed that degradation proceeded via methanethiol and formaldehyde, via an NADH-linked mono-oxygenase.

Methanethiol (MT) was further oxidized by methanethiol oxidase to formaldehyde, which was subsequently assimilated via the Serine pathway. It was not clear exactly what happened to the sulfur-moiety, except that sulfuric acid was detected in the medium.

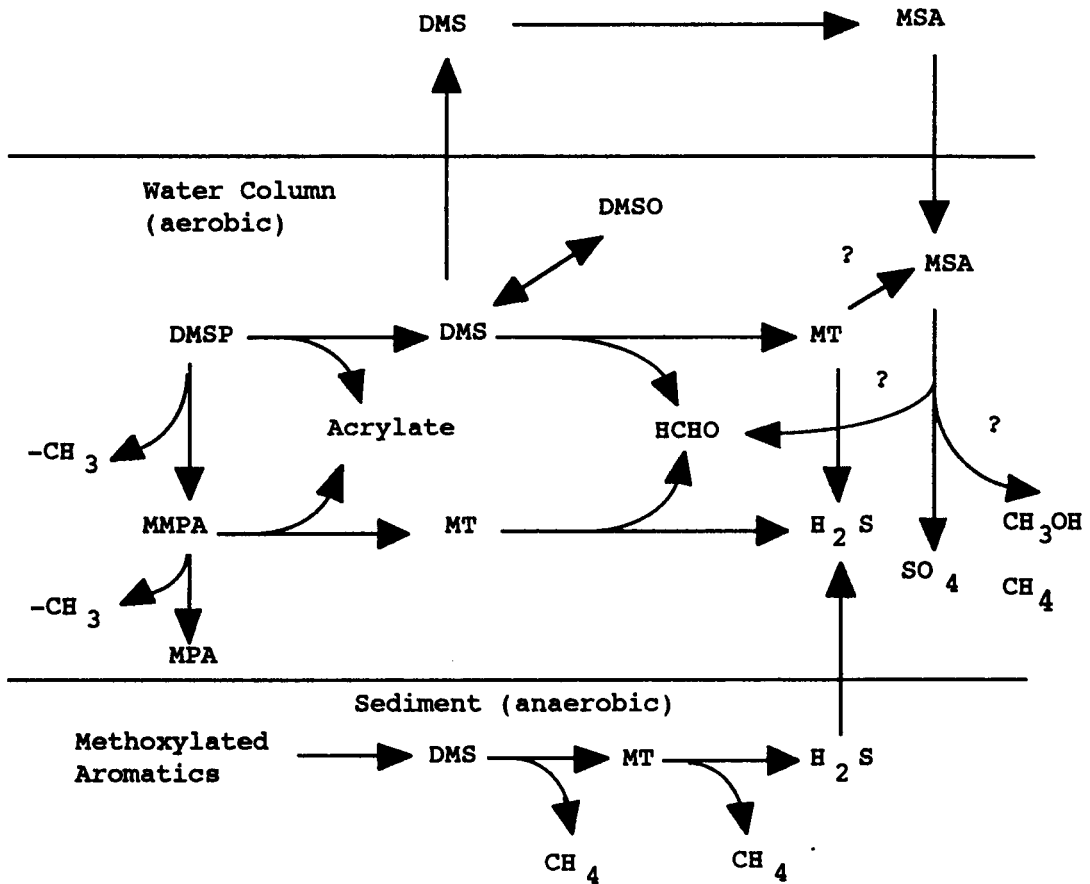
Interestingly, the authors reported that this strain was unable to utilise other C<sub>1</sub> substrates as carbon sources, including MSA. For *Thiobacillus thioparus* (Kelly and Smith, 1990), DMS was metabolized by the same pathway, except that formaldehyde is further oxidized, via formate, to carbon dioxide which was then assimilated into cell biomass, via RuBISCO.

DMS is also oxidized to DMSO photo-autotrophically by bacteria such as *Thiocapsa roseopersicina* and *Thiocystis* sp. in microbial mats (Visscher and van Gemerden, 1991). DMS acts as an electron donor for CO<sub>2</sub> fixation. In some species of *Thiocystis*, hydrogen sulfide is required.



Methanogens, from marine sediments, have also been identified as DMS-degraders. Ni and Boone (1991) isolated *Methanolobus sicilliae* from an oil well borehole. MT was also identified as an intermediate. MT was subsequently converted to hydrogen sulfide and methane. Kiene et al. (1986) also obtained an obligately methylotrophic estuarine methanogen in pure culture which utilised DMS. DMS was similarly degraded to MT, and then to hydrogen sulfide and methane.

The marine microbial conversions of DMS and related compounds are summarised in Fig. 1.6. This figure shows the flow of sulfur through the marine system, and the two significant carbon pools arising out of the biodegradation of DMSP and DMS. I have also included possible products of MSA oxidation. Formaldehyde and acrylate would probably be further oxidized or assimilated into cell biomass.



**Figure 1.6. Microbial metabolism of DMS and related compounds in seawater.** DMS; Dimethylsulfide, DMSP; Dimethylsulfoniopropionate, MT; Methanethiol, MMPA; 3-Methiolpropionate, MPA; 3-Mercaptopropionate. From; Kiene, 1991, Kiene et al., 1986, Visscher et al., 1992, Visscher and van Gernerden, 1991. Conversion of MT to MSA is speculative (Kiene, 1993, Blom and Tangerman, 1988).

### 1.2.3. Degradation of organic sulfur compounds.

Higgins et al. (1993) reported the biodegradation of monomethyl sulfate (MMS) in *Agrobacterium* and *Hyphomicrobium* species. A major structural difference between MSA and MMS is the absence of a C-S bond in MMS (CH<sub>3</sub>OSO<sub>3</sub>). Two pathways were proposed. In

*Hyphomicrobium* sp. MS72, a hydrolytic pathway occurred, where the initial step was the desulfurization of MMS and hydrolysis to give methanol, which was assimilated via conventional methylotrophic pathways (Ghisalba and Kuenzi, 1983). In *Agrobacterium* and *Hyphomicrobium* sp. MS223, biodegradation proceeded via a monooxygenation pathway, with MMS being initially monooxygenated to methanediol monosulfate, which was unstable and spontaneously breaks down to formaldehyde, with the release of sulfate. Strain MS223 expressed methanol dehydrogenase during growth on methanol, but not during growth on MMS.

Methanethiol (MT), as previously discussed, is a major product from the degradation of DMS and related compounds. MT can be further broken down to formaldehyde and hydrogen sulfide in hyphomicrobia and thiobacilli, and to methane by methanogens. Blom and Tangerman (1988), studying hepatic encephalopathy (caused by the toxicity of MT in the blood), found that whole blood (including the erythrocytes) metabolized MT to formate and sulfite. Small amounts of MSA were found (1% of total MT), suggesting that at least some of the MT is metabolized via MSA. MSA added to whole blood was not metabolized further. The major pathway suggested was the hydroxylation of MT, followed by dehydrogenation to formaldehyde. Interestingly, a small amount (0.5% of total MT) of MT was metabolized to DMS. The implication of this paper

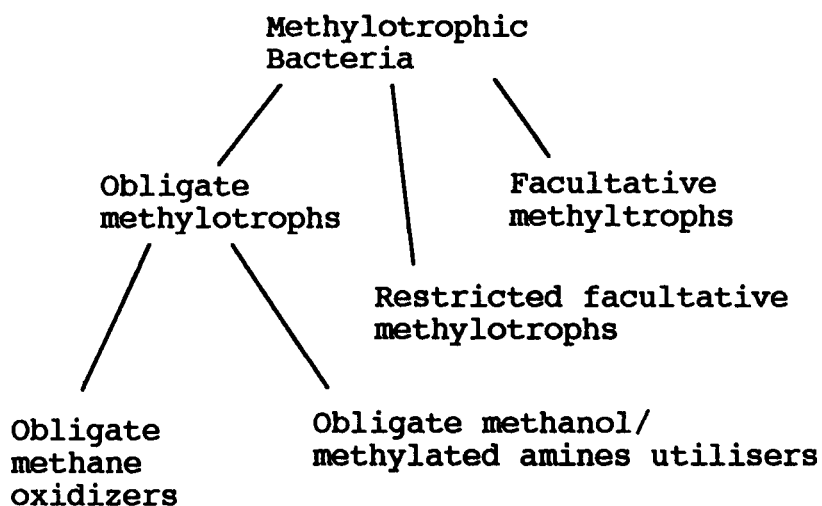
is that it seems perfectly feasible for there to be a biological source of MSA (from MT) in seawater, as well as a source from the photo-oxidation of atmospheric DMS.

### **1.3. METHYLOTROPHY.**

The methylotrophic bacteria are a diverse grouping of aerobic and anaerobic bacteria, characterised by their ability to utilise so-called C<sub>1</sub> compounds as their sole carbon and energy source. C<sub>1</sub> compounds, for the purposes of this discussion, are compounds with only one carbon, or no carbon-carbon bonds. These include methanol, the methylamines, methane, formate, DMS and MSA. I will be concentrating on a discussion of the characteristics of non-methane utilising methylotrophs, since the methane oxidising (i.e. methanotrophic), due to their specialist physiology, merit a separate discussion.

#### **1.3.1. Taxonomy of methylotrophs.**

Methylotrophic bacteria can be divided into three main subdivisions; facultative, obligate and restricted facultative methylotrophs. The sub-divisions of methylotrophic bacteria are illustrated in Fig. 1.7.



**Figure 1.7. The divisions of methylotrophic bacteria (from Green, 1992).**

Facultative methylotrophs are the most commonly described methylotrophs. They have the ability to utilise a broad spectrum of compounds, both  $C_1$ , and multicarbon substrates, such as sugars and organic acids. The obligate methylotrophs, on the other hand, can only metabolise compounds with no carbon-carbon bonds, with the exception of carbon dioxide. Their growth spectra on these compounds may also be restricted within this group of substrates. The classification of a third group of methylotrophs, the restricted facultative methylotrophs, is somewhat contentious. This group is defined as being bacteria only able to utilise a narrow range of multicarbon substrates (Green, 1992). Jenkins and Jones (1987) go on to further divide this group into the "restricted facultative methylotrophs" (those only able to grow on one or two multicarbon substrates) and the "less

restricted facultative methylotrophs" (those able to grow on a larger, but still limited, range of multicarbon substrates).

Methylotrophs are a diverse group of organisms, encompassing both gram negative and gram positive bacteria. In this respect, the facultative methylotrophs are not a true taxonomic group (Green, 1992), since their classification is made only on the basis of the ability to oxidise C<sub>1</sub> substrates. Nevertheless, a survey of these bacteria would be pertinent to this discussion.

#### 1.3.1.1. The gram-negative methylotrophic bacteria.

Facultatively methylotrophic bacteria can be divided into two groups; pink pigmented facultative methylotrophs (PPFMs) and non pigmented facultative methylotrophs.

The *Methylobacterium* spp. are a particularly well studied example of PPFM's. Examples of this genera are widely distributed in the environment. They are gram negative, strictly aerobic rods. Most produce a pink carotenoid pigment when grown on methanol. Other features of this group are they grow poorly or not at all on complex media such as nutrient agar. All reported strains assimilate carbon via the serine pathway. No facultative methanotrophic species has been isolated. Lynch et al. (1980) reported the

isolation of a facultative methanotroph, "*Methylobacterium ethanolicum*". Subsequently, it was shown that this was in fact a mixed culture (Lidstrom-O'Connor et al., 1983).

Another important group of gram negative facultative methylotrophs are the hyphomicrobia. As their name suggests, they are appendaged bacteria, often isolated from aquatic environments. They can grow on a variety of C<sub>1</sub> substrates such as methanol, formate, methylamines and dimethyl sulfide. They are more correctly described as restricted facultative methylotrophs since many seem incapable of growth on substrates with more than three carbons. Many show oligotrophic abilities, being able to utilise "carbon compounds in the atmosphere" (Green, 1993). Most marine facultative methylotrophs reported in the literature appear to belong to this group.

In addition to these there are a multitude of gram negative bacteria that defy any unifying classification, except that they are methylotrophic in terms of their biochemistry. They include *Paracoccus denitrificans*, strains of "*Pseudomonas*", *Rhodopseudomonas*, *Thiobacillus* and *Xanthobacter*.

*Paracoccus denitrificans* was first isolated in 1910 by Beijerinck and has since been extensively characterised. *P. denitrificans* is a gram negative hydrogen oxidising coccoid organism. Anaerobic

methylophilic growth on methanol is possible in this organism, using nitrate as the terminal electron acceptor. *P. denitrificans* possesses a methanol dehydrogenase with a very high similarity to *Methylobacterium* at the amino acid level (76% identity, Stouthamer, 1992).

The obligate methylophilic bacteria are a much more unified group, and include methane and non-methane oxidizing bacteria. The main genera of obligate methylophilic (non-methane oxidizing) are (at this time); *Methylobacillus*, *Methylobacter* (*M. methylovora*, *M. M15*, *M. P11*, *M. methanolica*, *M. methanophilus*, *M. clara*), *Methylophilus* and *Methylophaga* (Green, 1993). The genera *Methylobacillus* and *Methylophilus* are considered heterogenous. All are rods, and can be polarly flagellated or nonmotile. All assimilate carbon by the Ribulose Monophosphate (RuMP) pathway. Confusingly, some species of *Methylobacillus* and *Methylophilus* can utilise a limited range of sugars. The difference here is that the methylobacilli do not utilise glucose. Boulygina et al. (1993), by sequencing genes encoding for the 5S rRNA subunits, showed the presence of a third group within these two groups, provisionally called *Methylovorus*.

The genus *Methylophaga* is of some interest in the context of this discussion in that two described members of this group are both marine strains. Janvier et al. (1985) and Urakami and Komagata (1987) both



report that *M. marina* and *M. thalassica* cannot be distinguished by conventional means. Green (1992) reports some differences that can be determined by non-routine biochemical means (based on differing *R<sub>f</sub>* values for glucose-6-dehydrogenase and methanol dehydrogenase, obtained from PAGE experiments). Their differences have been determined by DNA homology data (Urakami and Komagata, 1987). Both can utilise fructose, but no other multicarbon compounds. Assimilation of carbon is via the RuMP pathway. The optimum salinity reported for *Methylophaga marina* and *Methylothaga thalassica* is about 3.0% (w/v) NaCl (Urakami and Komagata, 1987).

Methane oxidizing (i.e. methanotrophic) methylotrophs fall into three broad groups; types I, II and X. Type I and II differ in the arrangement of intracytoplasmic membrane ultrastructure, carbon assimilation pathways, fatty acid chain lengths and phylogeny. Type X have a similar internal membrane arrangement to Type I. Similarities extend to carbon assimilatory pathways, except that Type X methanotrophs can assimilate CO<sub>2</sub> autotrophically. Type X methanotrophs possess higher Mol% G+C DNA than Type I methanotrophs (Green, 1992, 1993). Bowman et al. (1993) classify Type X methanotrophs as Type I methanotrophs. The main methanotroph groups are *Methylococcus* sp. (Type X), *Methylocystis* sp. (Type II), *Methylobacter* sp. (Type I), *Methylomonas* sp. (Type I) and *Methylosinus* sp. (Type II) (Bowman et al., 1993). Not all species of

*Methylobacter* can oxidize methane, according to recent reviews (Green, 1992, 1993, Hanson et al., 1992).

Table 1.3. summarises the main characteristics of a selection of gram negative methylotrophs, including major groups of methanotrophs.

	Methylo- coccus	Methylo- monas	Methylo- sinus	Methylo- bacillus	Methylo- bacter	Methylo- phaga	Methylo- philus	Aceto- bacter	Para- coccus	Eypso- microbium
Cell Shape										
Rod	+	+	+	+	+	+	+	+	+/-	+
Coccus	+	-	-	-	-	-	-	-	+/-	+
cell dia. µm	1.0	0.5-1.2	0.6-1.0	0.3-0.5	0.8-1.0	0.2	0.3-0.6		0.5-0.9	0.2-0.3
motility	-	+	+	variable	+	+	variable	+	variable	-
Growth on:										
Methane	+	+	+	-	variable	-	-	-	-	-
Methanol	+	+	+	+	+	+	+	+	+	+
Methylamine	+	variable	-	variable	+	+	variable	-	+/-	+
Multicarbon substrates	-	-	-	variable	+	+	variable	+	+	variable
RMP pathway	+	+	-	+	-	+	+	+		
Serine pathway	-	-	+	-	+	-	-	-		
NaCl requirement	-	variable	-	-	-	+	-	-	-	
Oxidase	variable	+	+	-	+/-	+	+	+	+	
Catalase	+	+	+	variable	+	+	+	+	+	
PFB	-	-	variable	-	+		-		+	+
Pigment- ation	none	pink/white	white/ brown	white/pale yellow	pink- orange-red	pink	none		white/ yellow variable	
CO <sub>2</sub> fixation	+	-	-		-	-	-	-		-
Mol% G+C	59-66	51-59	63-67	50-56	49-60	43-49	50-53	51-65	65-68	59-65

Table 1.3. Characteristics of some gram negative methanotrophic and methylotrophic bacteria. Table compiled from Bowman et al. (1993), Green (1992, 1993), Brock and Madigan (1991), Hanson et al (1992), Lidstrom (1992).

PFB; Polyhydroxybutyrate, RMP; Ribulose Monophosphate Pathway, +/-; poor.

#### 1.3.1.2. The gram positive methylotrophic bacteria.

The third major group of facultative methylotrophs are the gram positive bacteria. These are the coryneforms, certain *Arthrobacter*, *Mycobacterium vaccae*, *Brevibacterium fuscum* 24, *Amycolatopsis methanolica* and certain *Bacillus* strains (Green, 1992, Arfman and Dijkhuizen, 1993). This group includes both mesophilic and thermophilic groups. This group has not been particularly well studied as regards their diversity. Assimilation of carbon in all the strains reported is by the ribulose monophosphate pathway (Green, 1993).

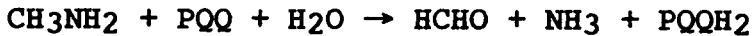
#### 1.3.2. The biochemistry of methylotrophic growth.

Despite the poor taxonomic position of the methylotrophs (see section 1.4.), their biochemistry is remarkably similar, especially for the gram negative bacteria, such as *Methylobacterium* and *Paracoccus denitrificans*. A number of common metabolic pathways and enzyme systems can be deduced. This section will describe the biochemistry of methylotrophy for the facultative and restricted facultative methylotrophs.

##### 1.3.2.1. Methylamine metabolism.

Gram negative methylotrophs, as seen earlier, can be characterised by their ability to grow using

methylamine as their carbon source. Methylotrophs able to grow on this substrate may possess methylamine dehydrogenase (MADH). This catalyses the reaction:



This enzyme is analogous to methanol dehydrogenase (MDH) in many respects. Similarly to MDH, it is a tetramer consisting of two alpha and two beta subunits (Davidson, 1993) and is located in the periplasm (Goodwin and Anthony, 1995). *In vitro* assays may be carried out using an artificial electron acceptor such as phenazine ethosulfate. *In vivo*, the natural electron acceptor is amicyanin, a copper bearing protein that mediates transfer of electrons from methylamine to the c-type cytochromes. The  $\beta$  subunits, unlike those in MDH, are bound covalently to a tryptophanyl tryptophanylquinone (TTQ) (McIntire et al., 1991) rather than to PQQ. The reaction mechanism of this enzyme has not been fully resolved (Davidson, 1993). Other primary alkyl amines can serve as substrates, but not diamines nor aromatic amines (Lidstrom and Chistoserdov, 1993). A wide range of methylotrophs possess this type of dehydrogenase, including obligate, facultative and autotrophic methylotrophs.

In addition, there are two further classes of methylamine oxidising enzymes. The first is a type of dehydrogenase found in restricted facultative

methylotrophs. This appears to differ from PQQ-methylamine dehydrogenases in that the electron acceptor is a c-type cytochrome (Burton et al., 1983). The second class are the methylamine oxidases. These are found in gram positive methylotrophs (Lidstrom and Chistoserdov, 1993) and catalyse methylamine according to the following scheme (taken from Anthony, 1982):



Methylamine oxidase (also known as amine oxidase) was first isolated and purified from *Arthrobacter* P1 (van Iersel et al., 1986) and found to contain the quinoprotein cofactor topaquinoxone (TPQ), bearing a copper atom. Little is known of the structure of this enzyme (Duine, 1993). However, no amine oxidase has been found containing PQQ.

#### 1.3.3.2. Methane oxidation.

Methane oxidation is only found in some genera of obligate methylotrophs. Methane is oxidized to methanol by methane monooxygenase (MMO). Methane monooxygenase (MMO) is NADH dependent with a relatively wide substrate specificity (Anthony, 1982). MMO is an enzyme complex consisting of three protein components giving mixed function (Dalton et al., 1993). The components required for activity are: the hydroxylase, A, the reductase, C and a component B. Component B is a regulatory protein. A (the site of

catalytic activity) is made up of three subunits,  $\alpha_2$ ,  $\beta_2$  and  $\gamma_2$  and two oxygen-bridged diiron clusters. The reductase, C, is a single polypeptide containing a FAD and an iron sulfide centre, to accept electrons from NAD(P)H to transfer to the hydroxylase. Component B contains no metal complexes (Froland et al., 1993).

There are two forms of MMO; particulate (membrane associated) MMO (pMMO) and soluble MMO (sMMO). The expression of either form depends upon the bacterial species and the concentration of copper in the growth medium, Soluble MMO is expressed by some methanotrophs (e.g. *Methylosinus trichosporium* OB3b, *Methylococcus capsulatus* (Bath)) in copper limited medium.

Particulate MMO is apparently present in all methanotrophs, though in the cases of *Methylosinus* spp. and *Methylococcus* spp., is only expressed in high copper medium (Murrell, 1992). The activity of this enzyme is in direct proportion to the copper: membrane protein in *M. capsulatus* (Chan et al., 1993). MMO has a very wide substrate specificity, able to oxidize a range of alkanes and halogenated aliphatics (Froland et al., 1993, Alvarez-Cohen, 1993).

The expression of sMMO has been well characterised (Murrell, 1992), though the expression of pMMO is poorly characterised. The sMMO gene clusters of *M. capsulatus* and *M. trichosporium* are broadly similar. Three genes (*mmo X*, *mmo Y* and *mmo Z*) encode for the subunits of component A. Protein C is encoded by *mmo C*

and component B is encoded by *mmo* B. In addition, *orf* Y encodes for a 12 kDa gene product, of unknown function (Murrell, 1992).

#### 1.3.2.3. Oxidation of methanol to formaldehyde.

Methanol dehydrogenase (MDH) is responsible in most methylotrophs for a one step oxidation of methanol to formaldehyde. The control of MDH biosynthesis is not fully understood. However Harms and van Spanning (1991) report three mechanisms to regulate MDH expression and activity:

[a] Product induction.

[b] Catabolite repression.

[c] Posttranslational modification. It has been observed that there may be a discrepancy between the amount of MDH detected immunologically and MDH activity (Harms and van Spanning, 1991).

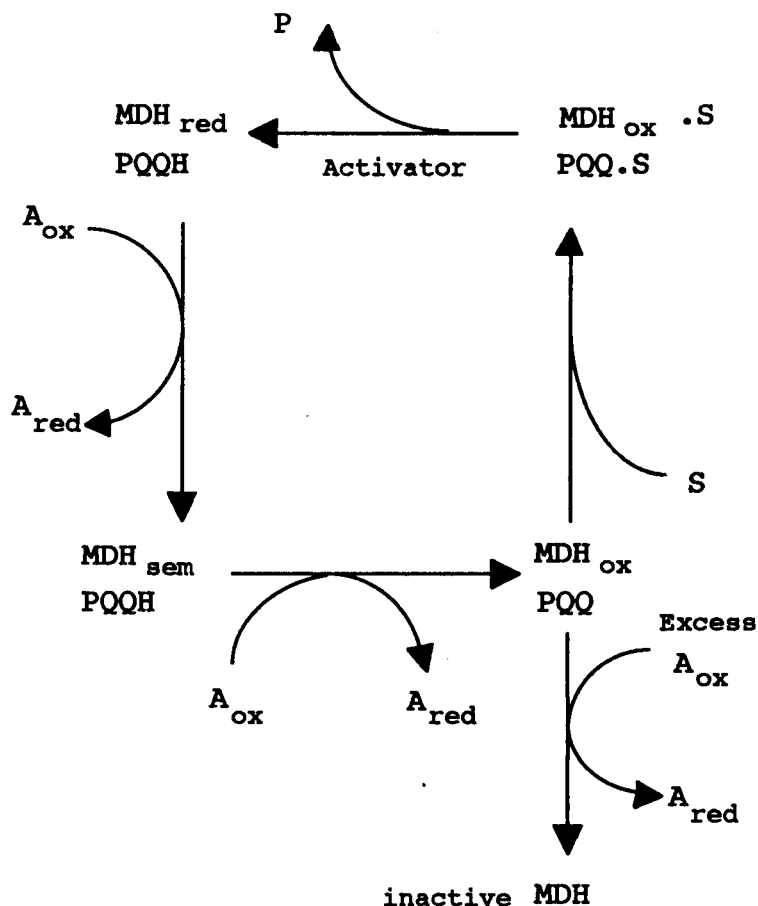
Goodwin and Anthony (1995) have reported at some length on the biosynthesis of MDH and methylamine dehydrogenase. Although the steps in MDH assembly are reasonably well understood, the regulation of MDH synthesis is not. In *Methylobacterium*, MDH appears to be expressed at low levels irrespective of the carbon source. Methanol induces MDH synthesis, but the presence of a second multicarbon substrate (in addition to methanol) inhibits synthesis of MDH (Goodwin and Anthony, 1995).



In *Paracoccus denitrificans*, the control mechanism appears more strictly regulated, and catabolite-repression occurs. Formaldehyde is thought to induce MDH.

MDH has a wide substrate specificity and is found in the periplasm (Goodwin and Anthony, 1995). This enzyme exists in close association with the electron acceptor system. This NAD<sup>+</sup> independent enzyme, methanol dehydrogenase (MDH) is remarkably conserved in mode of action. The molecular weight of MDH is around 149 kDa (Anthony, 1993). MDH occurs usually as a tetramer, (Anthony et al., 1993) of two  $\alpha$  and two  $\beta$  subunits.

Pyrrolo-quinoline quinone (PQQ) is the prosthetic group (Anthony, 1982). PQQ is not unique to MDH since many other dehydrogenases have since been found to contain PQQ. These include alcohol dehydrogenases from *Acinetobacter calcoaceticus*, glucose dehydrogenase from *Pseudomonas fluorescens* (cited Richardson and Anthony, 1992). The significance of PQQ manifests itself in the mechanism of MDH. Figure 1.8. shows a diagrammatic representation of the *in vitro* action of MDH and illustrates the four states of MDH that occur.



**Figure 1.8. Reaction cycle of methanol dehydrogenase**

(after Anthony, 1993). MDH<sub>sem</sub>; semiquinone form of MDH, MDH<sub>red</sub>; fully reduced form of MDH, MDH<sub>ox</sub>; fully oxidized form of MDH, A; one-electron acceptor (PES, PMS, Cyt C<sub>L</sub>). The activator is ammonia or an amine. S is substrate (i.e.. methanol). P is product (i.e. formaldehyde).

These states appear to be mediated by the redox states of PQQ, which are PQQ, PQQ<sup>-</sup>, and PQQH<sub>2</sub>. This has been shown to be dependant upon calcium ions bound within the PQQ-ααββ tetramer to maintain the correct configuration (Anthony, 1993). An electron acceptor, cytochrome C<sub>L</sub>, docks with the α subunit of MDH so activating the enzyme. The enzyme is hence active in

the oxidised form. The reaction mechanism has still not been fully elucidated. Anthony (1993) proposes two of the most likely mechanisms that have the most experimental support. In the first, reducing equivalents are transferred from methanol to PQQ, giving PQQH<sub>2</sub> and formaldehyde. The second mechanism provides a much more active role for PQQ. In this mechanism, PQQ complexes with the substrate at the active site of MDH prior to formation of PQQH<sub>2</sub>.

The function of the  $\beta$ -subunits is uncertain. Originally it was thought that these subunits were involved in the binding of PQQ. However amino acid sequence comparison with other dehydrogenases shows that this is unlikely to be the case. The  $\beta$  subunit however may be involved in some other way in the binding of PQQ to MDH (Anthony, 1993). It has also been suggested that the  $\beta$  subunit is directly involved in electron transfer, in that it induces a conformational change on the C<sub>L</sub> cytochrome, facilitating the strong redox behaviour of the enzyme.

The expression of MDH is not fully understood (Goodwin and Anthony, 1995). Nevertheless, structural genes have been identified in *Methylobacterium extorquens*, *Paracoccus denitrificans*, *Methylobacterium organophilum* and *Methylosinus trichosporium* (Goodwin and Anthony, 1995). They appear to be assembled into an operon; *mxoY* (regulatory), *mxoF* (encodes for the  $\alpha$ -subunit), *mxoJ* (encodes for third subunit of MDH, or

possibly a molecular chaperone), *mxg* (encodes for cytochrome  $c_L$ ), *mxI* (encodes for the  $\beta$ -subunit) and *pqqD* (encodes for the PQQ precursor). Peptides MxX and MxY activate the *mxF* promoter of the operon. Several other proteins are also involved in regulation, but their action is poorly understood. The assembly of MDH is also poorly understood. For a fuller discussion of MDH synthesis and regulation, refer to Goodwin and Anthony (1995).

#### 1.3.2.4. Oxidation of formaldehyde to formate.

Formaldehyde may be further oxidised to formate, and then to carbon dioxide. Methanol dehydrogenase has the ability to catalyse this reaction. Indeed, it has been reported that MDH biosynthesis may be induced by the presence of formaldehyde (Harms and van Spanning, 1991). This appears to be a mechanism to remove any accumulation of formaldehyde in the cell, since this is highly toxic to the cell. It has been observed that MDH may be expressed in large amounts in cells grown on C<sub>1</sub> substrates that do not in themselves produce methanol in the subsequent oxidation pathway (Kelly et al., 1994). This has been attributed to the accumulation of formaldehyde.

Methylotrophic bacteria may also produce formaldehyde dehydrogenases and aldehyde dehydrogenase. However, the presence of these enzymes is not a universal requirement for methylotrophs. The relationship

between these enzymes, substrate and MDH synthesis appears to be complex and largely unknown (Roitsch and Stolp, 1986). The formaldehyde dehydrogenases that have been characterised so far are NAD or NADP dependent (Duine, 1993). The cofactor may be PQQ (in the case of *Hyphomicrobium zavarzinii*) or NAD (formaldehyde dismutase in *Pseudomonas aeruginosa*). Little appears to be known of the mechanism of these enzymes.

Aldehyde dehydrogenases have also been found in some methylotrophs. As for the formaldehyde dehydrogenases, these are formate producing. The substrate specificity of these enzymes is uncertain, and to date, they have only been shown to display formaldehyde oxidising ability *in vitro*. However, it has been reported that aldehyde dehydrogenase isolated from *Methylococcus capsulatus* (Bath) can be transformed into a specific formaldehyde dehydrogenase by a modifier protein (S. Tate, personal communication.).

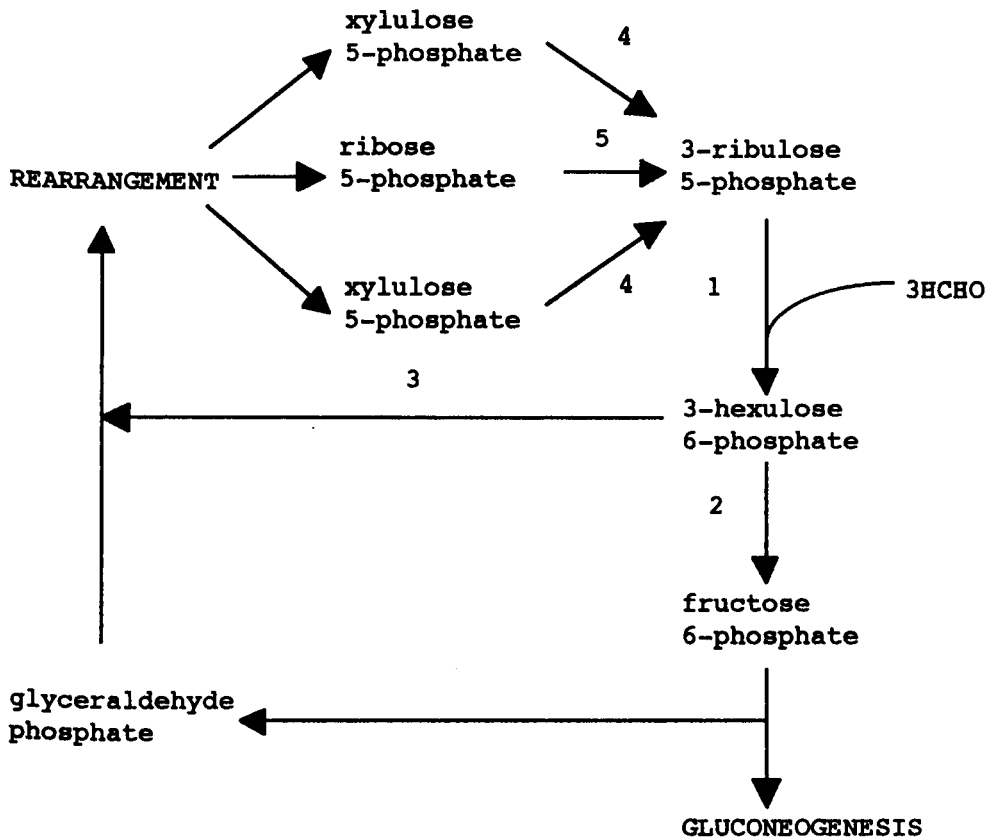
#### 1.3.2.5. Oxidation of formate to carbon dioxide.

Once oxidised to formate, there is a one step oxidation to carbon dioxide, carried out by formate dehydrogenase. Again not all methylotrophs will possess this enzyme (Anthony, 1982). They are typically NAD linked dehydrogenases. For many methylotrophs, formate dehydrogenase appears to be the sole enzyme for the regeneration of NADH during growth

on C<sub>1</sub> compounds. The ability of a methylotroph to utilise formate as a carbon source depends on the ability of the organism to assimilate CO<sub>2</sub>, either via RuBISCO or by using a carrier molecule such as methyl tetrahydrofolate.

#### 1.3.2.6. The RuMP cycle for the assimilation of formaldehyde.

The ribulose monophosphate (RuMP) pathway is found almost exclusively within the obligate methylotrophs. Cell carbon is assimilated at the level of formaldehyde (Anthony, 1982). Details of the pathway are not universally the same within those bacteria that possess it, but a number of features are conserved which allow a general approach to assaying for the presence of this pathway (Fig 1.9.). The ratios of xyulose 5-phosphate and ribose 5-phosphate produced depend on the particular cycle variant (KDPG aldolase/sedoheptulose biphosphate, fructose biphosphate aldolase/ transaldolase, fructose biphosphate aldolase/ sedoheptulose phosphate and KDPG aldolase/ transaldolase variants) (Anthony, 1982).



**Figure 1.9. Common features of the Ribulose monophosphate cycle for the assimilation of formaldehyde (based on Anthony, 1982).** Scheme shows key features common to all RuMP variants. 1; hexulose phosphate synthase, 2; hexulose phosphate isomerase, 3; hexulose phosphate isomerase, 4; pentose phosphate epimerase, 5; pentose phosphate isomerase.

It is only necessary to assay for two enzymes, hexulose phosphate synthase or hexulose phosphate isomerase, to show the existence or otherwise of the RuMP cycle.

Of the four variants, the KDPG aldolase/sedoheptulose bisphosphate variant has not been found in methylotrophs. The fructose bisphosphate

aldolase/transaldolase variant is rarely found (Anthony, 1982). The occurrence of the other two is neatly split between obligate methylotrophs and certain facultative (non-methane utilising) methylotrophs (Anthony, 1982). Note however, not all obligate methylotrophs possess the RuMP cycle (Table 1.4).

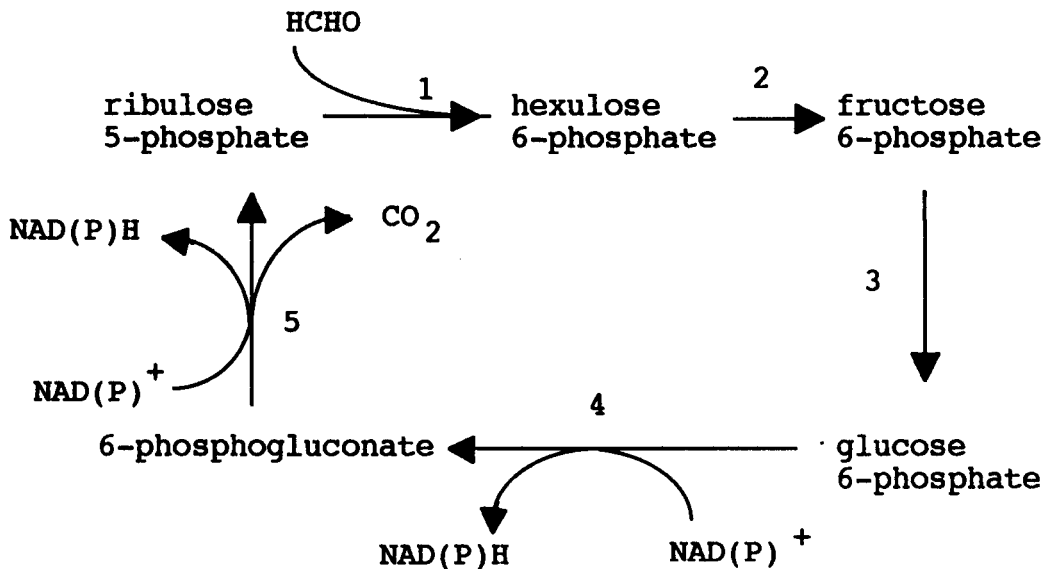
RuMP Variant	Cleavage phase	Rearrangement phase	Occurrence	Gluconeogenesis pathway
KDPG/TA	KDPG aldolase	transaldolase	mainly obligate methylotrophs	Entner/Doudoroff pathway
FBPA/SBPase	FBP aldolase	SBPase	mainly facultative methylotrophs	Glycolysis
FBPA/TA	FBP aldolase	transaldolase	some bacteria	Glycolysis
KDPGA/SBPase	KDPG aldolase	SBPase	not yet described	Entner/Doudoroff Pathway.

**Table 1.4. Summary of RuMP variants (reported and theoretical).** KDPG; 2-keto, 3-deoxy, 6-phosphogluconate, TA; transaldolase, FBPA; fructose bisphosphate aldolase, SBPase; sedoheptulose bisphosphatase.

Despite the variations, the RuMP cycles in these organisms still achieve the same end, namely to generate a molecule of a C<sub>3</sub> compound from three molecules of formaldehyde. These C<sub>3</sub> compounds, can then be further assimilated into the cell by "generic" oxidation pathways (Entner/Doudoroff and Glycolysis). The fourth pathway variant is a theoretical pathway with elements from the other variants, and would require enzymes found in both glycolytic and Entner/Doudoroff pathways. Anthony (1982) reviews the details of these pathway extensively.



Anthony (1982) also reports that this pathway can be used for the dissimilation of formaldehyde, i.e. the oxidation of formaldehyde to carbon dioxide in bacteria possessing Entner/Doudoroff pathway enzymes (Figure 1.10.).



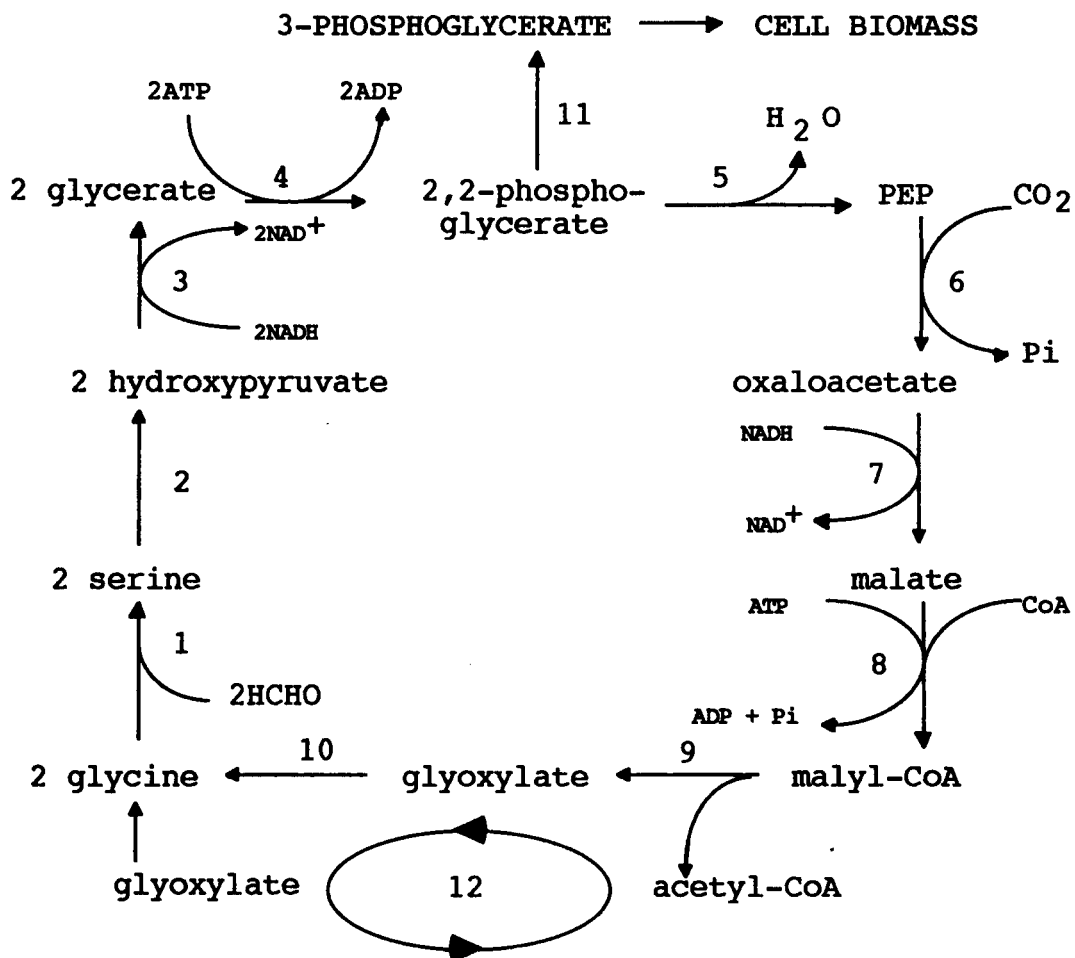
**Figure 1.10. Oxidation of formaldehyde to carbon dioxide via a dissimilatory pathway (after Anthony, 1982).** 1; hexulose phosphate synthase, 2; hexulose phosphate isomerase, 3; glucose phosphate isomerase, 4; glucose 6-phosphate dehydrogenase, 5; 6-phosphogluconate dehydrogenase.

In methylotrophs the main function of this is to provide reducing equivalents as NAD(P)H. For this pathway to exist, an active 6-phosphogluconate dehydrogenase is required (Anthony, 1982). Only methane oxidisers appear to be deficient in this respect.

### 1.3.2.7. Serine pathway.

The other pathway of formaldehyde assimilation/dissimilation is the serine pathway. Anthony (1982) includes an extensive discussion on this pathway in his review of methylotroph biochemistry. Most methylotrophs using this pathway are facultative. No non-methane utilising obligate methylotroph utilises this pathway. Some type II methanotrophs can utilise this pathway, e.g. *Methylosinus trichosporium*, *Methylocystis parvus* (cited Anthony, 1982). Unlike the RuMP cycle, the serine pathway is conserved in methylotrophs, with only two variants known (Figure 1.13.).

The two variants found are the  $icl^+$  (isocitrate lyase possessing) and  $icl^-$  variants. In  $icl^+$  variants, acetyl-CoA is oxidized to glyoxylate via a glyoxylate cycle. In  $icl^-$  variants (e.g. *Methylobacterium extorquens* AM1), since isocitrate lyase is not present, acetyl-CoA is oxidized to glyoxylate by some other pathway (Anthony, 1982). This difference has the end result in affecting overall growth yields (Anthony, 1982).



**Figure 1.11. The Serine pathway to assimilate formaldehyde (showing features common to  $icl^+$  and  $icl^-$  variants) (based on Anthony, 1982).** 1; serine transhydroxymethylase, 2; serine glyoxylate aminotransferase, 3; hydroxypyruvate reductase, 4; glycerate kinase, 5; enolase, 6; PEP carboxylase 7; malate dehydrogenase, 8; malate thiokinase, 9; malyl-CoA lyase, 10; serine glyoxylate aminotransferase, 11; phosphoglycerate mutase, 12; glyoxylate cycle (details not shown).

For diagnostic purposes, two enzymes can be assayed for convenience (see methods): hydroxypyruvate reductase and serine glyoxylate aminotransferase. The presence of both of these must be shown to occur in an

isolate before it can be classified as serine pathway positive. Hydroxypyruvate reductase activity occurs at low levels in RuMP positive methylotrophs, as well as in autotrophic methylotrophs such as *Paracoccus denitrificans* (Anthony, 1982). Serine transhydroxymethylase is the enzyme responsible for the addition of formaldehyde to glycine, to produce serine. However, this enzyme is extremely difficult to assay for routinely.

A key difference between this pathway and the RuMP cycle are that the intermediates are carboxylic acids not carbohydrates. A substantial portion of the carbon from formaldehyde is transformed to phosphoglycerate, whereby it is assimilated into cell material via phosphoenolpyruvate. Some phosphoglycerate remains within the serine cycle to maintain it.

#### **1.4. MARINE MICROBIAL ECOLOGY.**

Marine bacteria have been noted from the earliest days of bacteriology. Antoine van Leeuwenhoek had noted "many animalcules" in shellfish obtained in Holland (in and around the canals) (Payne, 1970) as early as the 1670's. In the 1930's and 1940's, Claude ZoBell isolated many marine heterotrophic bacteria, and investigated the effects of season on bacterial activity. However, study has been hampered by the specialist growth requirements of marine bacteria, making them difficult to cultivate in the laboratory.

Recent studies have shown that the marine environment is far more complex than had been assumed, with the potential for complex microbial habitats. A consideration of aspects of marine microbial ecology, in respect of distribution and physiological status should be made before embarking on a study to investigate microbial influences on the marine sulfur cycle.

#### **1.4.1. Bacterial distribution and activity in seawater.**

##### **1.4.1.1. Factors affecting marine microbial activity and diversity.**

The open ocean has been traditionally viewed as an oligotrophic environment, i.e. with low nutrient availability. Coastal areas, by receiving significant inputs of nutrients from land are more conducive for the survival of heterotrophic bacteria, but are highly variable (both in terms of nutrient inputs and physical processes). However, it has been known that deep oceans and seas are relatively stable environments (Fenchel, 1994), allowing vertical zonation to occur. This zonation is seen in the distribution of chemical species, such as oxygen, nitrate, and sulfate. The deeper the ocean, the greater the degree of zonation. Towards the surface, wind-driven turbulent mixing and ocean currents reduce this stratification. In shallow seas, such as the

North Sea, mixing affects all layers during the winter months. However, in summer, stratification is produced by the warming of the surface layers. This is discussed in some detail later.

#### 1.4.1.2. Distribution and activity of free-living bacteria.

The distribution and diversity of free-living bacteria had been very difficult to study until the advent of sensitive molecular biology techniques, such as PCR. Due to the perceived oligotrophic nature of the environment, it was difficult to obtain representative isolates from seawater samples. Estimates for total bacterial production were made using relatively crude chemical methods (in terms of expressing the microbial diversity of seawater, such as thymidine-incorporation) or by using microbiological techniques originally developed for clinical studies and for the analysis of drinking water (i.e. total counts using complex media such as Nutrient Broth/Agar). Some attempts have been made to quantify oligotrophic bacteria by cultural methods, such as dilution cultures (Schut et al., 1993), but given the potential metabolic diversity suggested by Sieburth (1993), such studies would invariably underestimate counts and diversity of free-living bacteria.

The nutritional status of free-living bacteria is uncertain. Clearly in coastal areas there is potential

for the existence of heterotrophic bacteria, given the nutrient inputs from land. It is difficult, however, to separate many of these bacteria from contaminating freshwater heterotrophic bacteria, particularly enteric bacteria.

Free-living bacteria in the photic zone (upper layers) of the sea are part of an overall community, which includes picoplankton, phytoplankton and zooplankton. This has been termed the "microlitresphere" (Fogg, 1988). Generally, the numbers of each of these constituents are (per ml);  $10^6$  heterotrophic bacteria,  $10^4$  picoplanktonic phototrophs and  $10^3$  nanoplanktonic predators (Fogg, 1988, estimated counts using epifluorescence microscopy). Normally, these numbers are fairly constant, and are not affected by temperature, nutrient status or salinity (Fogg, 1988).

A balance is maintained, and the cells appear to grow normally at a rate of growth with doubling times in hours in their natural environment (Fogg, 1988). Nutrient elements, such as nitrogen and phosphorous, are cycled rapidly. The exchange of materials is almost exclusively by molecular diffusion. The effects of steepened diffusion gradients and the high surface to volume ratio of bacterial cells ensures that nutrients are supplied at a sufficient rate to ensure a maximum rate of growth.

The microbial population of the photic zone is particularly responsive to changes in conditions, and, since the energy of the system is derived from the activities of the phytoplankton, to diurnal light changes (Fogg, 1988). During the winter, especially in shallow seas, the increased mixing leads to a reduction in photosynthesis (the phytoplankton being such that they do not receive much light most of the time), and corresponding nutrient availability in the community. The bacteria appear to enter a viable but non-culturable state (Nilsson *et al.*, 1991). In spring, activity returns when phytoplankton start photosynthesis again. It is likely that these bacteria have novel survival mechanisms.

It is generally agreed that most of the marine bacterial population is metabolically active (Schut *et al.*, 1993). However, the nature of the mode of nutrition (heterotroph vs. oligotroph) is in some dispute. Horowitz *et al.* (1983) found that euryheterotrophy rather than oligotrophy was characteristic of bacteria isolated from oligotrophic environments (Alaska). Bacteria isolated using high-nutrient media were more specialised in their mode of nutrition than the dominant euryheterotrophs (isolated on low nutrient media). Schut *et al.* (1993), in a similar pristine environment (also Alaska) determined that over 50% of isolates obtained, using dilution cultures, were obligate oligotrophic bacteria. It seems that the enrichment technique used is critical



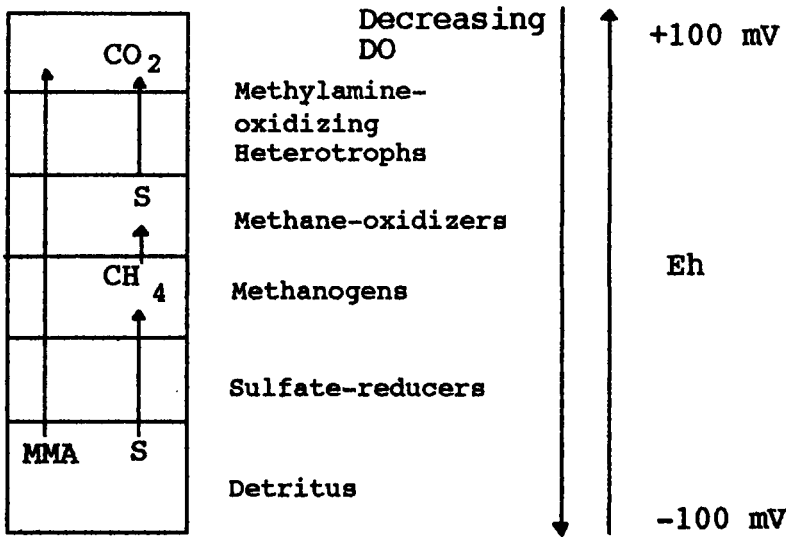
in assessing the diversity of a marine microbial community, given that two similar areas gave differing results.

A far more successful method to assess diversity of free-living bacteria is by using molecular biology techniques. Typically, this involves obtaining the total DNA of a sample, amplifying and cloning the 16S rRNA gene sequence, followed by sequencing (Fuhrman et al., 1993). Diversity can be assessed by comparison of the sequences obtained with those in 16S rRNA gene databanks. Unfortunately, this technique is of limited use in functional studies, as 16S rRNA sequence does not relate necessarily to biochemical function. Methods to enumerate specific members of a bacterial population would require the use of functional gene probes, in addition to the 16S rRNA route. By implication, such a group must already be well studied and reasonably coherent. An excellent example of this is the current research to enumerate marine methane-oxidizing bacteria. This particular group has been well studied at the biochemical level. The key enzyme involved in methane oxidation, methane monooxygenase, is also well conserved in methanotrophic bacteria, so allowing the development of a method to specifically enumerate methanotrophs in the environment, without a cultivation step (J.C. Murrell, personal communication). With the advent of mRNA probes, such an approach could be applied to assess activity of these bacteria *in situ*.

#### 1.4.1.3. The distribution and activity of attached bacteria.

The presence of particulate matter (e.g. decomposing plant or animal matter) in the water column can also have a profound effect on bacterial ecology. Such material would be rich in carbon, and offer a stable environment for bacteria. Griffith et al. (1994) demonstrated that bacteria attached to such detritus were more active on a per-cell basis than free-living bacteria, although free-living bacteria were responsible for the bulk of bacterial production. It was found that, in addition, the "quality" of the substrates in the particulate affected bacterial activity. The authors conclude that although attached bacteria constitute only a small fraction of the total bacterial population, they may be responsible for a significant fraction of phytoplankton decomposition and oxygen uptake.

Not only can these particles offer enhanced substrate sources, but also they can (potentially) provide reduced microniches. It has been postulated that anoxic microniches may be generated within the oxic water column by bacterial reduction (Sieburth 1993, Sieburth et al., 1993)) on and around algal particulates (Fig. 1.12.).



**Figure 1.12. Marine bacterial microniche proposed by Sieburth (1993).** Model shows section through a pellet composed of detrital material and bacterial communities acting in symbiotrophy. MMA; methylamines, S; unspecified substrates. DO; Dissolved oxygen.

Initially aerobic bacteria digest the algal debris (in this paper, it was suggested that these were methylamine oxidizing methylotrophic bacteria), so consuming dissolved oxygen. Anaerobic microniches would then be formed, allowing colonisation by oxygen tolerant methanogens (found in the water column), so producing methane, and allowing the establishment of methane-oxidizing bacteria. Dissolved oxygen levels would be reduced to new levels, whereupon the sulfate-reducing bacteria become established. After each successive colonisation, the bacteria types would be displaced according to their relative affinities for oxygen. The end result would be a layered microhabitat. The heterotrophic and methylamine oxidizing bacteria, the most aerophilic, would be

outermost, followed by relatively microaerophilic methanotrophic bacteria, supplying nutrients to these bacteria, followed by methanogens and the innermost layer being the sulfate-reducing bacteria (Sieburth, 1993, Sieburth et al., 1993).

Sieburth reports that these microniches are very fragile and that the above model is based on a number of field observations. It appears that these microniches are very difficult to study under laboratory conditions. Presumably, these niches are transient, and would cease to function once the algal detritus is completely metabolized (so halting carbon flow).

#### **1.4.2. Some effects of physical and chemical processes on bacterial distribution and activity.**

##### **1.4.2.1. Distribution of bacteria in the subsurface microlayer.**

Bacteria are found throughout the water column and in the sediments. However, there are situations which may affect distribution. These situations are, for the most part, a product of physical events. For instance, the surface microlayer, comprises the top few centimetres (in theory, the top 20Å, but this is difficult to sample (Williams et al., 1986)). The microlayer is known to possess special chemical properties. Many proteins, amino acids,

polysaccharides and fatty acids form stable surface films; their concentrations are thus potentially many times higher in the surface microlayer than surrounding water masses (Williams et al., 1986). There is the potential in this region for enrichment of bacteria. In open ocean samples, Williams et al. (1986) found that bacterial carbon in the surface film was 3-4 times more than that of the same body of water sampled at 10 cm<sup>3</sup> depth. In coastal waters, there was little difference. In this situation however, they found that the numbers of "metabolizing" bacteria (determined from on-board mesocosm experiments) was considerably lower in surface films. Closer examination of the data showed, however, that although a smaller percentage of the surface bacteria were metabolically active than in subsurface populations, on a per cell basis, they displayed higher metabolic activity. The implication here is that surface bacterial communities play a major role in nutrient turnover in the oceans, and so constitute a significant active population.

1.4.2.2. The effects of water interfaces on bacterial distribution and activity (thermoclines, nutrient availability, light availability, oxygen availability and riverine inputs).

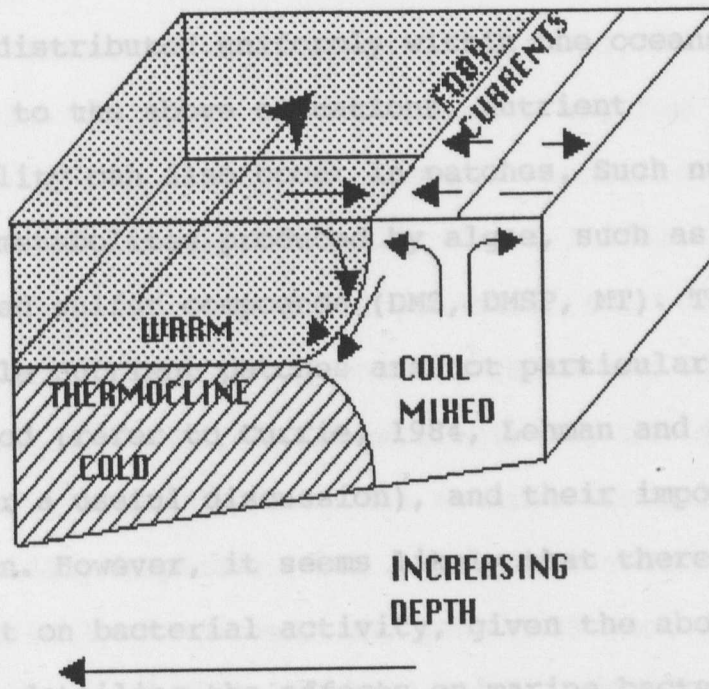
The distribution and activity of bacteria in the water column is also subject to other physical influences.

Fogg (1988) describes how sea fronts influence the

microbiology of seawater. Sea fronts are formed at the interfaces of water masses. On a grand scale, the Antarctic Convergence provides an example; cold northerly flowing water dips below warmer water. The interface of Antarctic water and Atlantic water leads to temperature, nutrient and salinity gradients being set up. On a smaller scale, sea fronts can be set up during the mixing of river water and seawater, and the formation of thermoclines, in shallow seas such as the North Sea (during summer). The thermocline sea fronts have the most obvious effect on biological activity. A typical situation is illustrated in Fig. 1.13. The sea front consists of three components; a mixed cold body of water (i.e. river outflow), a warm stratified top layer and a cold bottom region.

When a thermocline is set up (i.e. in summer, due to increased temperatures), transport of nutrients from the depths is restricted. Similarly, nutrient inputs from riverine inputs (i.e. cold water) are also restricted. The result is that surface algal blooming rapidly exhausts nutrients, so growth at the surface is restricted. Along the warm water-cold water interface, increased growth results, since light is sufficient, but there is a sufficient flow of nutrients here from the depths. In the cold depths, mixing occurs, so although there are plenty of nutrients, growth is restricted by a lack of light. Increased bacterial cell densities have been found at the surface just on the stratified front side (warm

water) from the estuarine water (cold). A possible explanation is that surface eddy formation (observed as estuarine waters meet seawater) results in some transfer of nutrients (Fogg, 1988).



**Figure 1.13. Schematic three-dimensional diagram of a shallow-sea tidal mixing front (after Fogg, 1988).**

Vertical circulation indicated by small arrows. Large arrow indicates along-front flow.

Similar "fronts" can also be found in deep seas, where an interface can be formed between oxic and anoxic layers. Zubkov et al. (1992), for example, report on a semi-permanent boundary at the oxic and anoxic interfaces in the depths of the Black Sea, leading to the establishment of a unique microplankton community. Thus, it can be seen that the physical processes

observed in front formation can affect both bacterial metabolic activity as well as distribution.

#### 1.4.2.3. Microscale nutrient patches.

It appears, contrary to expectations, that substrates are not distributed uniformly within the oceans. In addition to the above situations, nutrient availability can also occur in patches. Such nutrients include metabolites produced by algae, such as methylated sulfur compounds (DMS, DMSP, MT). These microscale nutrient patches are not particularly well understood (refer to Currie, 1984, Lehman and Scavia, 1984, for a useful discussion), and their importance uncertain. However, it seems likely that there will be an effect on bacterial activity, given the above examples detailing the effects on marine bacteria by enhanced substrate availability.

#### 1.4.3. Isolation of marine methylotrophic bacteria.

Few *bone fide* marine methylotrophic bacteria have been reported in pure culture, mainly due to the general cultivation problems associated with marine bacteria. Marine methanotrophs are better represented. Several strains have been obtained in pure culture (Lees et al. 1991, Holmes et al., 1995). Endosymbiotic methanotrophs have also been reported as apparently existing in the gills of marine invertebrates (Cavanaugh, 1993), though these have not been



successfully cultivated. As already discussed, because MMO is relatively conserved, further novel species can be retrieved using molecular techniques. For other methylotrophs it is more difficult. All marine methylotrophs to date have been gram negative (Urakami and Komagata, 1987, Strand and Lidstrom, 1984,) and most are described as obligate or restricted facultative methylotrophs. The reason why few have been obtained is not clear. Seawater contains innumerable C<sub>1</sub> compounds (Kelly et al., 1993, Kiene, 1993), so there is clearly a potential for these bacteria to be relatively common. There are few functional molecular tools available for the study of marine methylotrophs, with the notable exception of methanol dehydrogenase (MDH). The genes encoding for MDH are highly conserved. The gene *MxaF* (encoding for the  $\alpha$ -subunit of MDH) has been used to detect methylotrophic bacteria in a wide range of environmental samples (McDonald et al., 1995). However, it seems likely that conventional enrichment methods currently remain the most effective way to study the diversity of these bacteria. Given that the activity and numbers of bacteria are affected on the micrometer level by the occurrence of sea front, microniches, surface microlayers, nutrient patches and other phenomena, it is important the correct sampling conditions and techniques are applied to maximise the diversity of bacteria enriched for.

### 1.5. AIMS OF WORK PRESENTED.

The aims of this project were:

[i]. To enrich and isolate MSA-oxidizing bacteria in pure culture from a wide variety of marine and freshwater sites at different times of the year.

[ii]. To characterise purified cultures using traditional microbiological methodologies.

[iii]. To determine the pathway of MSA mineralization, principally in the marine isolates, using both *in-vitro* and *in-vivo* biochemical techniques.

[iv]. To initiate the molecular characterization of the bacterial strains obtained, in order to successfully enumerate MSA oxidizing bacteria in the environment.

## CHAPTER 2: MATERIALS AND METHODS.

### 2.1. MATERIALS.

#### 2.1.1. Chemicals and reagents used.

Standard chemicals used were of analytical quality from Sigma, Merck-BDH and Aldrich. Methanol used in biochemical studies was "Aristar" grade (BDH). Gelrite was sourced from Merck (Kelco Division, San Diego) and Phytigel from Sigma.

#### 2.1.2. Preparation of dilute methanesulfonic acid (MSA) solution.

MSA was obtained from Elf-Aquitaine (Paris, France) as the 99% free acid. For routine use, 5 M neutral solutions were prepared by careful addition of sodium hydroxide solution, to give the sodium salt.

Commercially available supplies of MSA are contaminated with acid-base indicators (of unknown type), necessary during the production process. To remove them, the 5M solution was passed through activated charcoal powder, and filtered to remove aggregates. The MSA solution was then autoclaved at 121°C for 15 min, and stored at room temperature

### 2.1.3. Preparation of methanol-free formaldehyde.

Most commercial formaldehyde preparations contain methanol as a contaminant, which is unsuitable for kinetic studies. Methanol-free formaldehyde solution was prepared by autoclaving a solution of paraformaldehyde [40 % (w/v)] in 3 ml heat-sealed glass ampoules for 4 hr at 121°C. The subsequent hydrolysis yielded a formaldehyde solution of around 40 % (w/v).

### 2.1.4. Media.

#### 2.1.4.1. Media used for enrichment and routine cultivation of marine bacteria.

Enrichments and subculturing were carried out in marine ammonium mineral salts media (MAMS), with phosphate buffering, containing the following (in g per litre of double-distilled water):  $(\text{NH}_4)\text{SO}_4$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.2; NaCl, 30.0; FeEDTA, 0.005;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.002;  $\text{KH}_2\text{PO}_4$ , 0.36;  $\text{Na}_2\text{HPO}_4$ , 1.91; trace elements solution (Whittenbury et al., 1970), 1ml  $\text{l}^{-1}$ ; vitamin solution (Kanagawa, 1982), 1ml  $\text{l}^{-1}$ , and the carbon source as described in chapters 4 and 5. Phosphate, trace metal and vitamin solutions were sterilised separately to the mineral salts medium. Trace metal and vitamin solutions were both filter-sterilised before use. Phosphates were autoclaved before use.

Other media used (in enrichments only) were artificial sea salts (Sigma), MNMS (similar to MAMS, with the substitution of  $1.0\text{g l}^{-1}$   $\text{NH}_4\text{NO}_3$  for  $\text{NH}_4\text{SO}_4$  and aged sea water (autoclaved, filtered sea water, with the addition of a 10X ammonium mineral salts basal medium (AMS ) to give a final concentration of 1X AMS). The AMS medium used consisted of (g per litre double distilled water):  $(\text{NH}_4)\text{SO}_4$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.2;  $\text{FeEDTA}$ , 0.005;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.002;  $\text{KH}_2\text{PO}_4$ , 0.36;  $\text{Na}_2\text{HPO}_4$ , 1.91; trace elements solution (Whittenbury et al., 1970),  $1\text{ml l}^{-1}$ ; vitamin solution (Kanagawa, 1982),  $1\text{ml l}^{-1}$ .

#### 2.1.4.2. Medium used for enrichment and routine cultivation of freshwater bacteria.

Freshwater isolates were cultivated using MinE basal salts medium (Owens and Keddle, 1969): (g per litre of double- distilled water);  $(\text{NH}_4)_2\text{SO}_4$ , 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.165;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.05;  $\text{KH}_2\text{PO}_4$ , 0.624;  $\text{K}_2\text{HPO}_4$ , 1.2.

Trace element solution and vitamins were added as previously described for MAMS basal salt medium.

Phosphates were autoclaved before addition to the main salts solution.

2.1.4.3. Media used for routine cultivation of type strains used in comparative studies.

*Rhodopseudomonas sphaeroides* was cultivated using the medium described by Lueking et al. (1978). *Pseudomonas diminuta* was grown using the medium described by Ballard et al. (1968). *Thiobacillus versutus* was cultivated using MinE (refer to section 2.1.4.2), with the addition of 8 ml l<sup>-1</sup> 1M NaOH. The carbon source used was 25 mM monomethylamine. *Methylococcus capsulatus* (Bath) was cultivated using the medium described by Whittenbury and Dalton (1981)

#### 2.1.5. Buffers.

For suspension of cells for standard biochemical analysis and short-term storage (c. 1-2 weeks at -80°C), 40 mM Tris-HCl (pH 7.0) with 3.0% (w/v) NaCl was used. Cell-free protein extracts were prepared and diluted in 40 mM Tris-HCl (pH 7.0). Cell stocks, for long term storage, used a 20 mM phosphate buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub> and 20 mM Na<sub>2</sub>HPO<sub>4</sub> mixed to a final pH of 7.0).

#### 2.1.6. Chemical Analyses.

##### 2.1.6.1. Estimation of methanol by gas chromatography.

Methanol was detected in culture supernatants by flame ionisation gas chromatography (Pye-Unicam Series

204 linked to a Hewlett-Packard Integrator) using a Poropak Q column, under the following conditions: nitrogen; 30 ml min<sup>-1</sup>, air; 0.5 kg cm<sup>-3</sup>, hydrogen; 1.0 kg cm<sup>-3</sup>, oven temperature; 180°C, injector temperature; 200°C, detector; 250°C.

#### 2.1.6.2. Measurement of sulfite in aqueous solution using Ellman's Reagent.

Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid) (Johnston et al. (1975)) was used to determine sulfite production in culture supernatants, cell-free extracts, in agar/ Phytigel plates and in whole cell suspensions. For whole cell studies, 30-90 mg (dry weight) of cells were inoculated into 25ml of MAMS containing 50 µmol MSA, and incubated at 30°C. 1.5 ml samples were taken over a 3 hour period and centrifuged for 10 min at 10,000 g. 0.1 ml of a solution of Ellman's Reagent (1g per litre of 0.1M phosphate buffer (pH 7.0) was added to 1 ml of supernatant, and the absorbance measured at 412 nm after 5 to 10 min (to allow the reaction to go to completion).

#### 2.1.6.3. Estimation of Formaldehyde.

The concentration of formaldehyde in solutions prepared from paraformaldehyde (section 2.1.3.) was assessed using the method of Nash (1953). The assay reagent consisted of (in a final volume of 1 litre of

double distilled water): 2ml acetyl acetone, 3ml acetic acid and 150g ammonium acetate. 2ml of assay reagent was incubated with 500 $\mu$ l of sample for 45 min at 37°C. A yellow colour subsequently developed, which could be quantified spectrophotometrically at 412 nm. The concentration of formaldehyde in the sample was determined by using a standard curve prepared using "Analar" formaldehyde/ methanol solution.

## **2.2. SAMPLING PROGRAMME.**

### **2.2.1. Sampling methods.**

Samples of sea water and sediment were obtained from a number of sites at various times of the year. A standard methodology was established, to ensure reproducibility. 50 to 100 litre nylon plastic carboys or 2 litre brown glass Winchester bottles were used throughout for the collection of sea water samples. The containers used in all cases were washed thoroughly in double distilled water, and then two to three times at the sampling sites, before filling. Minimal headspace was provided before sealing the containers for transportation. Where possible, samples were obtained at high tide (to reduce the risk of contamination by terrestrial bacteria). Samples were then stored at 4°C until further analysis, though in practice enrichments were carried out as soon as possible (usually within 12-24 h of sampling).



### **2.2.2. Sampling Sites.**

Both freshwater and marine samples were obtained for enrichments. Freshwater samples were obtained from Finham Sewage Works (Stoneleigh, Warwickshire) and from a pond near the National Agricultural Centre (Stoneleigh, Warwickshire). Seawater samples were gathered from a variety of sites around the coast of the British Isles, as well as from a number of off-shore locations. Sites were chosen to give as much variability in terms of the habitat sampled. These sites are described in some detail in Chapter 3.

### **2.3. ENRICHMENT METHODS.**

#### **2.3.1. Sample preparation.**

Seawater was first prefiltered through a glass fibre filter, of nominal pore size 1.2  $\mu\text{m}$  (Whatman GF/C), to remove suspended particulate matter and zooplankton. The seawater fraction could then be enriched for bacteria. A number of methods were adopted to enrich the MSA-oxidizing bacterial population within the filtered sample.

#### **2.3.2. Direct batch enrichment.**

1000 x MNMS (to give a final concentration of 1 x MNMS) was added to 0.5 to 1 litre of prefiltered sea water in a 2 l flask. A carbon source was added and

the flasks were incubated at 15, 20, 25 and 30°C on shaking incubators at 100 rpm. Static enrichments were also carried out. Carbon sources used were MSA, monomethylamine chloride, methanol, methane, taurine, isethionate and cysteate. Carbon sources were added at an initial concentration of 12.5 mM, alone or in combination with 12.5 mM MSA. 25 mM MSA was also used as a sole carbon source for enrichments.

### **2.3.3. Chemostat enrichment.**

Sea water was used as described as above, but inoculated into a 1 litre fermenter (LH Engineering), fitted with pH, aeration and temperature control. The phosphate source was provided by 2 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> (phosphate buffer was not used). Artificial sea-salts were also used as a substitute for MNMS in some enrichments. Both MSA and methane were simultaneously used as carbon sources. Methane was continuously supplied, at a rate of 40 ml min<sup>-1</sup> and air at a rate of 150 ml min<sup>-1</sup>. Small additions of MSA (5 mM) were made at intervals when required, i.e. when the pH of the culture started to drop (which was used as a presumptive indicator of MSA utilization).

### **2.3.4. Filter enrichments.**

1 to 2 litres of seawater was filtered through a 1.2 µm filter as previously described in section 2.3.1. The filtrate was then filtered onto 0.22 µm glass

fibre filters (Millipore) and the filters inoculated into 2 l flasks containing MNMS and a carbon source as previously described in section 2.3.2 . Incubation temperatures were as described in section 2.3.2.

#### **2.3.5. Enrichment using large volumes of seawater.**

This was similar in principle to that described for the enrichment in section 2.3.4., except that much higher volumes of seawater were processed. Using a tangential flow filtration apparatus (Millipore "Pelican"), 50-100 litres of sea water was reduced to a volume of 500-1000 ml, giving a relatively high cell density, which provided a suitable inoculum. Two different sized filters were used; 0.2  $\mu\text{m}$  and 1000 kDa. The concentrated cell suspension was then diluted 1 in 2 with 2x MNMS, and a carbon source added as described in section 2.3.1.

In order to test whether the efficiency of the apparatus in retrieving the methylotrophic population, 10x MNMS (giving a final concentration of 1 x MNMS) and 25 mM methanol was added to the all the seawater filtrates. If growth resulted, this would indicate that methylotrophic bacteria were present in the filtrate, showing that the particular size of filter was ineffective in retrieving these bacteria from seawater,

### 2.3.6. Agar tube enrichments.

In order to enrich for bacteria that may be sensitive to high levels of oxygen, "sloppy" agar tube enrichments were set up. Sloppy agar was prepared using 0.5 % (w/v) Noble agar (final concentration) in MAMS. 1 to 2 litres of sea water were filtered as described in section 2.3.4. onto 0.22  $\mu$ m filters. The bacterial cells collected were then resuspended in 10 ml of 3.0% (w/v) NaCl solution, and added to sterile test-tubes. The sloppy agar media was then added and mixed to a final concentration of 0.5% (w/v). The test-tubes were then aseptically stoppered with foam bungs to allow a free flow of air. Bacterial isolates could then be separated by their relative affinities for oxygen; growth occurred as "rings" in the agar, which could be removed using a Pasteur pipette. Ellman's reagent was added to the agar (1 ml per litre of agar, solution prepared as described in section 2.1.6.2.) to indicate the production of sulfite (by producing a yellow halo around any developing colonies), and thus acted as a presumptive indicator of MSA utilisers.

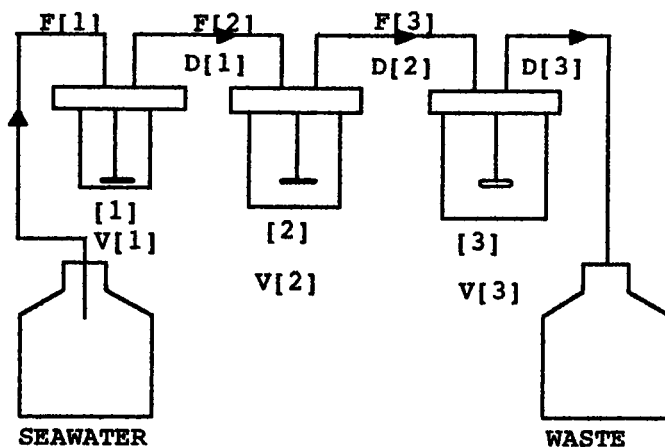
### 2.3.7. Gradostat enrichment.

Mixed cultures can be separated, on the basis of their growth rates, using a gradostat fermenter system. In this case, the gradostat used was a series of interconnected fermenter vessels (1 litre nominal

volume, LH Engineering), of increasing working volume (set by altering the height of the overflow). The first fermenter (volume 500 ml) in the series was filled with seawater, with 1000x AMS (to a final concentration of 1x AMS) and 25mM MSA added, from a carboy, fed at a constant flow rate. The overflow was connected to the next fermenter in the series (volume 750 ml). A third fermenter (volume 1000 ml) completed the series. Although the "medium" flow rates into each fermenter were identical the volumes varies, so the dilution rates varied. At steady state, using Monod kinetics (see Sinclair, 1987 for a fuller explanation);

$$D = \mu \text{ (where } \mu \leq \mu_m), \quad D = \frac{\text{Flow Rate}}{\text{Volume}}$$

The more slowly growing constituent of a mixed enrichment culture may, therefore, be separated from others by careful selection of dilution rates (faster growing bacteria would be washed out where dilution rate is more than  $\mu_m$ , but retained where the dilution rate is less than  $\mu_m$ ). Figure 2.1. illustrates the volumes and flow rate used in the particular enrichment carried out.



**Figure 2.1. Gradostat apparatus to enrich for marine bacteria.**  $V[1] = 500 \text{ ml}$ ,  $F[1] = 15 \text{ ml hr}^{-1}$ ,  $D[1] = 0.03 \text{ hr}^{-1}$ ,  $V[2] = 750 \text{ ml}$ ,  $F[2] = 15 \text{ ml hr}^{-1}$ ,  $D[2] = 0.02 \text{ hr}^{-1}$ ,  $V[3] = 1000 \text{ ml}$ ,  $F[3] = 15 \text{ ml hr}^{-1}$ ,  $D[3] = 0.015 \text{ hr}^{-1}$

## 2.4. ISOLATION OF PURE BACTERIAL CULTURES.

### 2.4.1. Criteria for a pure culture.

The purity of cultures was determined by a combination of phase-contrast light microscopy ( $\times 1000$ ), growth on complex medium and colony examination (using a plate microscope).

Cultures were judged pure when both colonies on plates and cells, when viewed microscopically, appeared uniform. Purity was also determined by examination of growth on a range of solid media (i.e. MAMS + agar, Nutrient agar, MAMS + Phytigel).

#### 2.4.2. Serial Dilution.

Primary enrichments were assessed for presumptive growth on MSA by measuring the rise in optical density (600 nm) and by microscopic observation of cultures where turbidity was low. Where growth was poor, but observed microscopically, the entire enrichment was centrifuged at 10,000 g, and the cell pellet resuspended (in 40 mM Tris-HCl/ 3.0% (w/v) NaCl) to a volume giving an appreciable turbidity (e.g. OD<sub>600</sub> of 0.2). Serial dilutions of all enrichments were prepared by inoculating flasks of MAMS, MNMS, AMS or NMS (media prepared as described in section 2.1.4), depending on the enrichment culture (freshwater or marine), with inocula of 0.5%, 1%, 5%, 10% and 20% v/v, and incubating at 30°C. 25 mM MSA was provided as the carbon source. The flasks with the smallest inoculum showing growth was further subcultured.

#### 2.4.3. Solid culture media.

Following the liquid sub-culturing procedure described above, it was desirable to obtain discrete colonies on a solid-surface medium. Marine methanotrophic organisms are known to be difficult to cultivate on agar-based media (Lees et al., 1991). Also routine preparations of agar may contain unspecified carbon sources. A range of gelling agents was used to screen both the semi-purified sub-cultures, (described in 2.4.2, and the primary enrichments described in

section 2.3), for MSA utilisers. The following gelling agents were used:

a. "Bactoagar" (Difco). This was incorporated into mineral salts media at a concentration of 2.0% (w/v).

b. "Noble Agar" (Difco). This purified agar was used at the following concentrations (w/v); 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1.0%, 1.5%, 2.0%.

c. "Phytigel" (Sigma). This highly purified bacterial gellan gum (Kang et al., 1982) is also known as Gelrite (Merck-Kelco) and "Gel-Gro" (ICN-Flow). This gum gives excellent handling properties and is very suitable for cultivation of slow-growing microorganisms, since it does not dry out quickly. A solution of Phytigel (1.0 or 2.0% (w/v)) was prepared by carefully dissolving Phytigel in boiling distilled water, followed by immediate autoclaving at 121°C for 15 min. Once solidified (at 60°C), Phytigel formed a permanent gel. After autoclaving, the solution was mixed with an equal volume of a prewarmed (60°C) solution of double-strength MAMS. Phosphates, vitamins, trace elements and carbon source(s) were added so that the final concentration of Phytigel was 0.5 or 1.0% (w/v). Gelling was accelerated by the addition of the salts solutions. This was probably due to the presence of magnesium ions, which are known to aid the polymerisation process (Noble and Gow, 1993).



d. Silica Gel. Silica gel petri dishes were prepared the method of Parkinson et al (1989).

#### **2.4.4. Separation of isolates by oxygen gradient.**

Sieburth (1993) has suggested that bacteria in the marine water column could exist in oxygen-limited micro-habitats found in suspended particulate matter. A method was devised to try and enrich for and separate microaerophilic populations of bacteria. Previous experiments had shown that use of 0.5% (w/v) Noble agar allowed cultivation of marine bacteria. 100  $\mu$ l of the inoculum (either sea water or primary enrichment) were added to 10 ml sterile test tubes and 8 ml of autoclaved MAMS, with 0.5% (w/v) Noble agar, or 0.25% (w/v) Phytigel added. 20 mM MSA provided the carbon source. The test-tube was then aseptically stoppered by a foam bung, to allow the free transmission of air. Bacteria of differing affinities for oxygen would be separated into growth bands within the agar. Since 0.4% (w/v) agar was used, these growth bands could be removed with a Pasteur pipette for further subculturing.

#### **2.5. ROUTINE CULTIVATION OF ISOLATES.**

##### **2.5.1. Flask cultivation.**

Once isolates were obtained, by differing enrichment techniques, the basic nutritional requirements were

determined; vitamin requirement, temperature and nitrogen source. MAMS medium (containing 3.0% (w/v) NaCl) was used routinely for cultivation and subculturing marine bacteria. Freshwater and terrestrial isolates were cultivated in AMS medium. Cultivation was carried out in 250 ml flasks, with a 100 ml volume. This was found to give the highest biomass yields. All isolates were cultivated at 30°C, on a rotary flask shaker (at 100 rpm).

#### **2.5.2. Petri dish cultivation.**

Marine isolates were routinely grown on Phytagel plates (1.0% (w/v)) with MAMS. Freshwater isolates were grown on Noble agar plates (2.0% (w/v)) with AMS (both at 30°C).

#### **2.5.3. Fermenter cultivation.**

In order to obtain high yields of cells for biochemical studies, fermenter cultures were used. Growth of all isolates was carried out in 12 and 5 litre fermenter vessels (LH Engineering, Stokes Poges, UK), fitted with pH, temperature and oxygen control. The fermenter was run in a "fed-batch" mode; i.e. MSA was added in aliquots to a final concentration of 40 mM. Additions were made when oxygen consumption (measured by an *in-situ* oxygen electrode probe) stopped dropping, indicating that the substrate (MSA) had been completely consumed by the culture. Use of 3M

KOH (2M KOH used for 5 l fermenters) for pH control was found to give higher growth yields than 3M NaOH. Use of a concentrated alkali was necessary in large volume fermenters due to pump flow rate limitations; 2M KOH could not be added at a high enough rate to counteract falling pH during exponential growth in cultures of OD<sub>600</sub> greater than 0.7.

## **2.6. MICROSCOPY.**

### **2.6.1. Light microscopy.**

Routine light microscopy was carried out using a Kyoga-Unilux III (Tokyo) phase-contrast microscope. Light photomicrographs were produced using a Zeiss Axioskop (Zeiss, FRG) phase-contrast microscope (1000x magnification) fitted with a video camera (Hamamatsu CCD C4742) and image analysis facilities. Images were captured on video, and still images produced using Fluorovision (Improvision, UK) software, operating on an Apple-Macintosh Quadra 840AV. Images were sharpened and cropped using Graphic-Converter v2.06 (US). These completed images were then outputted on a high resolution printer (600 dpi laser printer).

### **2.6.2. Scanning electron microscopy.**

Scanning electron microscopy was carried out using a Jeol JSM-T330A microscope, with a non-cryogenic stage. Cells for microscopy were washed twice in 40 mM Tris/

3.0 % (w/v) NaCl (pH 7.0), and then fixed in glutaraldehyde (2.5% (w/v)) for 4 h. Cells were then washed in double distilled water, to reduce salt content. Cells were resuspended to a density similar to that of the original culture, i.e. typically to an optical density of (at 600 nm) of 0.25. For mounting, 25 mm aluminium stubs (Agar Products) were used. Silver-containing paint ("Electrodag", Acheson Colloids (USA)) was applied to the surface of a stub, and a clean 20 mm circular glass coverslip placed on top. This was allowed to dry thoroughly. 100  $\mu$ l of the fixed cell suspension (in water) was applied to the coverslip, and allowed to dry overnight in a vacuum dessicator. A gold coat was applied to the surface for 100 s in a sputter coating machine (Biorad E5200 Autosputter Coater). Photographs were taken using a Polaroid-type instant camera, using Polaroid "53" and "55" film.

## **2.7. PHYSIOLOGICAL CHARACTERISATION OF ISOLATES.**

### **2.7.1. Growth conditions studies (in liquid media).**

Salinity growth profiles of marine isolates were constructed by studying the effects of varying the sodium chloride content of MAMS, from 0% to 5.0% (w/v) NaCl (typical seawater concentrations are in the order of 3.0 to 3.5% (w/v) NaCl). Single colonies picked from Phytigel plates were inoculated into 25 ml flasks (containing 10 ml MAMS). Growth was measured by

regular measurements of the OD<sub>600</sub>. Optimal salinity was defined as the salinity concentration that allowed the fastest growth rate over a 7 day period.

Other simple studies included vitamin requirement, nitrogen source (nitrate, ammonium or nitrogen gas as a source), and the effects of varying the pH of the phosphate buffer. Phosphate buffers of different pH were devised using the tables generated by Sørensen (anon., 1970). The effects of temperature on the rate of growth were also determined at 15, 20, 25 30 and 37°C.

#### **2.7.2. Growth rate studies.**

The growth rates of all the isolates obtained from the enrichment programme were determined, along with the growth yields, for a range of substrates; MSA, methanol, mono-methylamine and acetate. These values were determined by inoculating (5% v/v) flasks containing 12.5 mM of the carbon source with MSA grown cells. At intervals, samples were taken and the OD<sub>600</sub> measured. For determination of molar growth yields, 10 ml samples were taken during the stationary growth and "death" phase (this was determined where the change in OD<sub>600</sub> started dropping). It was assumed when this phase was reached, all of the substrate had been consumed. These samples were filtered onto pre-weighed nitrocellulose filters (0.22  $\mu$ m pore size, Millipore GC), which were then dried in an oven (80°C) until a

constant weight was reached. It was recognised that in the case of MSA, large pH changes could affect both the growth yield and growth rate. True molar growth yields on MSA were determined from fermenter-grown cultures as previously described. In this case, MSA was judged to be completely consumed once the oxygen consumption rate dropped (determined from oxygen electrode measurements).

### **2.7.3. Substrate growth profile.**

The ability to utilise C<sub>1</sub>-substrates (substrates with no C-C bond), sugars, organic acids and various organosulfonates as a sole source of energy and carbon was tested by addition of 20 mM of substrate to preinoculated 20 ml universal vials. Dimethyl sulfide (DMS) was added directly to the vials to a final concentration of 5 mM, because of its known toxic properties (Suylen, 1988). Growth was assessed after 7 to 10 days by measuring the optical density at 600 nm. In all cases, samples of the positive cultures were plated onto nutrient agar plates to check for evidence of contamination (contaminants were likely to grow on nutrient agar, whereas the marine isolates did not).

### **2.7.4. Physiological Characterisation.**

Isolates obtained from the enrichment programme were tested for the following: Gram stain, capsule stain, oxidase, catalase, following the

protocols described by Smibert and Krieg (1990). Cytochrome oxidase was tested for using diagnostic strips (Merck "Bactodent"). An exhaustive range of API-type tests was not carried out, as previous work with similar methylotrophic bacteria had showed that most of these would be redundant (Kelly *et al.*, 1994).

## **2.8. BIOCHEMICAL CHARACTERISATION OF ISOLATES *IN-VIVO*.**

### **2.8.1. Calibration of the oxygen electrode.**

Oxygen uptake profiles for isolates TR3, PSCH4, FW2 and FW6 were determined using a Clarke-type oxygen electrode (Rank Bros. Ltd) linked to a chart recorder (Gallenkamp Euroscribe). To determine absolute oxygen-uptake values, it was necessary to calibrate the electrode for each of the buffers used, according to the phenazine methosulphate (PMS) linked method described by Robinson and Cooper (1970). The method was slightly modified, with the substitution of phenazine ethosulphate (PES) for PMS.

The reaction mixture consisted of (in a 3 ml volume) 20  $\mu$ g PES (phenazine ethosulphate, a substitute for PMS and 800 units Catalase (Sigma), dissolved in the buffer used for the particular experiment being carried out (see section 2.7.5.2). This mixture was added to the water-jacketed chamber of the oxygen electrode (temperature held at the experimental temperature, 30°C), and saturated with oxygen, using a

small fish-tank type air pump, for 20 min., and stirred. During this time, the electrode was disconnected, and the chart recorder set to 0%. Once the reaction had reached a steady state, the electrode was reconnected, and the recorder set to 100%. 10  $\mu$ l aliquots of freshly prepared NADH solution (14 mg ml<sup>-1</sup>) were added at intervals, and the resultant oxygen consumption measured on the chart recorder.

#### **2.8.2. Determination of oxygen: substrate stoichiometries.**

Substrate: oxygen stoichiometries were determined using a Clarke-type oxygen electrode as described in section 2.8.1. Cells were grown to a high density in a fermenter, and harvested by centrifugation at 10,000 g, at 4°C. These cells were washed at least four times in 1000 times their pellet volume using a Tris-HCl buffer. For the freshwater isolates studied, this buffer was 40 mM Tris-HCl (pH 7.0), and for marine strains, 40 mM Tris-HCl/ 3.0% (w/v) NaCl (pH 7.0) was used. Repeated washings were required to remove all traces of MSA and any metabolites that were present. After the final wash, the cells were resuspended in 1/100th the original culture volume. Cells were used immediately or drop frozen in liquid nitrogen, and stored at -80°C until required. If cells were stored, upon thawing, they were washed a further two times, to remove debris from any lysed cells.



For O<sub>2</sub>-substrate stoichiometry experiments, the 3 ml O<sub>2</sub>-electrode cell contained the following; 50-100  $\mu$ l cell suspension (30-50 mg dry weight), air-saturated Tris buffer (as described) and a carbon source. All experiments were carried out at the normal incubation temperature of 30°C, with the temperature being controlled by a Churchill thermal-circulator. The oxygen electrode was allowed to run for 1-2 min to ascertain the endogenous oxidation rate. Substrate was added by micro syringe through the top of the electrode cell. Substrate: oxygen consumption ratios were determined using a range of substrate concentrations (0.05 to 10  $\mu$ mol per assay). Cells were kept on ice until required. If the endogenous oxidation rate was very high (i.e. almost indistinguishable from the rate due to substrate oxidation), it was necessary to incubate the cells at 30°C for 2-3 h. Cells were often used repeatedly, by sparging the experimental mix with air prior to addition of fresh substrate. There was no effect on the stoich-iometry obtained, and overall oxidation rates were reproducible.

### **2.8.3. Determination of substrate oxidation spectrum.**

Oxidation rates of a range of C<sub>1</sub> compounds and alkyl-sulfonates (methanol, formaldehyde, formate, MSA, ethanesulfonate, propanesulfonate, butanesulfonate, taurine, isethionate, cysteate, dimethylsulfide) were determined using the oxygen electrode, used as

previously described. 20  $\mu$ mol of substrate was tested on each occasion, in triplicate.

## **2.9. BIOCHEMICAL CHARACTERISATION OF ISOLATES IN-VITRO.**

### **2.9.1. Preparation of cell-free extracts.**

Fermenter-grown cells were harvested by centrifugation at 10,000 g for 15 minutes, and washed 4 times in 40 mM Tris-HCl/ 3.0% (w/v) NaCl (pH 7.0) buffer. The cells were resuspended in  $1/100$ th volume 40 mM Tris-HCl (pH 7.0). These cell slurries were passed twice through a chilled French-press minicell at 137 MPa, followed by centri-fugation at 20,000 g for 1 hour at 4°C, to separate cellular debris. The clear supernatants were stored until required by drop-freezing in liquid nitrogen and long term storage at -70°C. Some deterioration in certain enzyme activities could be expected after several months storage, but the enzymes to be assayed for, were known to be reasonably stable (with the exceptions of MSA oxygenase, the stability of which was unknown, and RuBISCO).

### **2.9.2. Determination of total protein content.**

The Biuret assay, described by Herbert et al. (1971) was found to give the best combination of reproducible results and convenience when compared with the Biorad

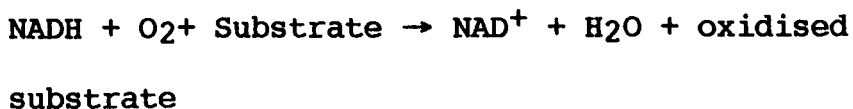
and Lowry methods. Protein concentration (in mg ml<sup>-1</sup>) was determined spectrophotometrically at three different dilutions of the extract being tested. Standards used were BSA (Sigma) based protein standards sets.

### **2.9.3. Polyacrylamide gel electrophoresis of cell-free extracts.**

Cell free extracts obtained from all the isolates and strain M2 (described by Baker et al, 1991) were compared by SDS-acrylamide gel electrophoresis. 13.5 % (w/v) SDS-polyacrylamide gels were prepared using standard methods (Laemmli, 1970). Gels were photographed on a light-box using a Minolta SLR-type camera, fitted with a zoom-lens. The film used was Kodak Tmax-100 film.

### **2.9.4. Assay of MSA oxygenase activity.**

The oxidation of MSA by cell-free extracts of marine strains was determined spectrophotometrically by a variation of the assay described by Kelly et al. (1994). This assay utilises the reaction:



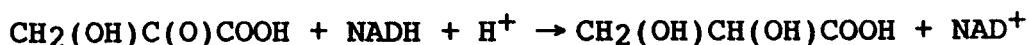
The reaction mix consisted of the following (in 1 ml); 35.6  $\mu\text{mol}$  TRIS-HCl (pH 7.0), 1.0  $\mu\text{mol}$  NADH (freshly

prepared in water), 0.2–0.5 mg of protein. The reaction was initiated with 0.25–2.5  $\mu\text{mol}$  of MSA, after the endogenous NADH oxidation rate was measured for 1–2 min, at 340 nm. All NADH oxidation rates were measured on a Beckman DU-70 spectrophotometer, at 30°C. The blank used was 35.6  $\mu\text{mol}$  TRIS-HCl (pH 7.0) in 1ml of water. The negative controls used were boiled extract (of the strain being tested) and *Methylobacterium extorquens* AM1 (grown on methanol), which cannot utilise MSA. Three separate determinations were made, using a range of MSA concentrations. It was found that activity decreased rapidly, once the extracts were thawed (even when stored on ice). Hence, only 50  $\mu\text{l}$  of extract was thawed at a time, to achieve consistent results.

#### **2.9.5. Assay of formaldehyde assimilation pathway enzymes.**

##### **2.9.5.1. Hydroxypyruvate reductase assay.**

Hydroxypyruvate reductase (HPR) is a key enzyme in the Serine pathway. HPR was assayed for by the method described by Crema and Lidstrom (1990). This spectrophotometric assay utilises the reaction:

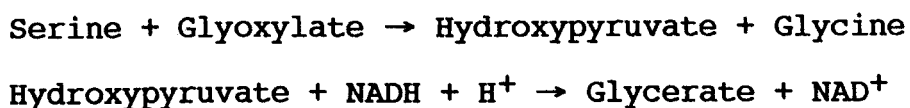


where hydroxypyruvate is reduced to D-glycerate by hydroxypyruvate reductase. The reaction mix consisted

of (in 1 ml): 500 mM sodium phosphate buffer (pH 7.5); 100 $\mu$ l, 38 mM ammonium sulphate; 100 $\mu$ l, 4mM NADH (freshly prepared solution); 100 $\mu$ l, and 500  $\mu$ l of double-distilled water. 0.5-1.0 mg of extract (in a volume of 100 $\mu$ l) was added, and the endogenous rate measured. To determine the apparent  $K_m$  and  $V_{max}$  for this enzyme, variable amounts of 2.0mM lithium hydroxypyruvate reductase were added (10 to 200  $\mu$ M), with the volume of water adjusted accordingly, to make a final volume of 1.0 ml. Unlike the assay described by Krema and Lidstrom (1990), the assay was carried out routinely at 30°C. Rates were determined by subtracting the endogenous rate from the total rate. Three separate determinations of the  $K_m$  and  $V_{max}$  values were made for each of the isolates, from extracts obtained from MSA-grown cells. The positive control used was extracts of *Methylobacterium extorquens* AM1 (grown on methanol), with the assay also carried out at 30°C.

#### 2.9.5.2. Measurement of serine-glyoxylate aminotransferase activity.

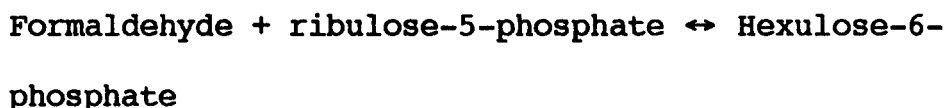
Serine-glyoxylate aminotransferase (SGA), along with HPR, is a key enzyme in the Serine pathway. The assay used the protocol described by Goodwin (1990). The assay utilises the following series of reactions:



The positive control used extracts prepared from *Methylobacterium extorquens* AM1 (grown on methanol), with the assay being carried out according to the protocol previously described (Goodwin, 1990).

#### 2.9.5.3. Determination of hexulose phosphate synthase activity.

Hexulose phosphate synthase (HPS) is a key and unique enzyme in the ribulose monophosphate cycle and is easily assayed using  $^{14}\text{C}$ -labelled formaldehyde. The enzyme catalyses the following reaction:



HPS was assayed for in marine MSA-utilizing bacteria using the cell-free assay described by Kelly and Wood (1984). Cell-free extracts were prepared from MSA and methanol grown cells, as described in section 2.8.1. A positive control was devised by using cell-free extracts prepared from methane-grown *Methylococcus capsulatus* (Bath) (assayed at 45°C). Two negative controls were provided by substituting water or boiled extract for the cell-free extract.

#### **2.9.6. Methanol dehydrogenase assay.**

Methanol dehydrogenase activity was assayed for as described by Day and Anthony (1990). This is a dye-linked spectrophotometric assay, utilising phenazine ethosulphate (PES) as a primary electron acceptor. A second dye, 2, 6-dichloroindophenol (DCPIP), was included to provide a second coupling reaction that could be measured spectro-photometrically at 600 nm. An activator, ammonium chloride, was included in the assay mix.

Cell-free preparations of methanol-grown *Methylobacterium extorquens* AM1 were used as a positive control. Activities were determined measuring the rate obtained 30 seconds after starting the reaction, using PES. A high ammonia-dependant endogenous rate is common in many MDHs, and is ignored in determining activities (Day and Anthony, 1990).

#### **2.9.7. Formate dehydrogenase assay.**

Formate dehydrogenase catalyses the final step in the complete oxidation of a C<sub>1</sub> compound. As previously discussed, formaldehyde may be assimilated into cell biomass by a variety of means. Formaldehyde may be further oxidized to formate by one of a number of formaldehyde dehydrogenases. Complete oxidation is reached by the conversion of formate to carbon dioxide, by formate dehydrogenase:



The assay used followed that described by Jollie and Lipscomb (1990), and is based upon the detection at 340 nm of NADH produced. The positive control used was cell-free extracts prepared from methane-grown *Methylococcus capsulatus* (Bath) (assayed at 45°C), and the negative control was boiled cell-free extract.

**2.9.8. Measurement of ribulose 1,5-bisphosphate carboxylase (RuBISCO) activity in cell-free extracts.**

RuBISCO is responsible for the fixation of carbon dioxide to two molecules of 3-phosphoglycerate. The rate of fixation of CO<sub>2</sub> by RuBISCO was estimated by examining the fixation of <sup>14</sup>CO<sub>2</sub> in cell-free extracts, as described by Kelly and Wood (1982). Cell-free extract was prepared from freshly harvested cells, and the extract used immediately as previously described in section 2.8.1. A negative control was provided by using boiled extract (of the strain being tested). The positive control was cell-free extracts prepared from monomethylamine-grown *Thiobacillus versutus*.



## 2.10. MOLECULAR CHARACTERISATION OF ISOLATES.

### 2.10.1. Preparation of chromosomal DNA from strains TR3 and PSCH4.

Chromosomal DNA was prepared according to the protocol modified from that described by Oakley and Murrell (1988). Where there was significant RNA contamination of DNA preparations, 5  $\mu$ l of RNase (10 mg ml<sup>-1</sup>) was added to degrade RNA.

The sedimented DNA was thoroughly dried in a dessicator, and resuspended in 10 ml of TE (Tris-EDTA buffer; 50 mM Tris-HCl, 1 mM NaEDTA, pH 7.4). This solution was added to 23 ml of TE, containing 33 g of caesium chloride, and 2 ml of ethidium bromide solution (10 mg ml<sup>-1</sup>), in a polypropylene Oakridge tube, which was then heatsealed (with no air gap). The tube was centrifuged at 100,000 g overnight at room temperature. The purified DNA could be visualised in the tubes under ultra violet light, as a luminous band. The band was carefully extracted using a wide bore needle, taking care not to disturb other cellular debris and protein on the side of the tube. Ethidium bromide was removed by repeated extraction using isopropanol, until no pink coloration remained in the aqueous (DNA containing) phase. DNA was reprecipitated from the aqueous phase, using chilled absolute ethanol, followed by storage at -20°C for 1 to 2 h. Nucleic acids were sedimented by centrifugation and

ethanol removed by washing the pellet in chilled 70% ethanol. Purified DNA was stored as a dried pellet at -20°C.

#### **2.10.2. Visualisation of chromosomal and plasmid DNA.**

0.7- 1.2% (w/v) agarose gels were used to visualise DNA using standard techniques. The quality of DNA preparations was established using the restriction enzymes *EcoRI* and *BamHI* to cut the DNA.

#### **2.10.3. Determination of mol% G + C content of DNA.**

The mol% G + C content of DNA from marine strains was determined by the spectrophotometric method described by Frederiq et al. (1961). Standards used were DNAs prepared from *Escherichia coli* B (mol% G+C = 51), *Clostridium perfringens* (mol% G+C = 24) and *Micrococcus luteus* (mol% G+C = 70).

### CHAPTER 3: ENRICHMENT AND ISOLATION OF MSA OXIDIZING BACTERIA FROM A VARIETY OF AQUATIC ENVIRONMENTS.

#### 3.1. INTRODUCTION.

The physiological status of the bacteria in a sample is important when considering enrichment strategies. In particular, for the marine bacteria, the concept of viable, but "nonculturable", bacteria had to be taken into account. Some of these bacteria may prove to be totally unculturable simply by being removed from their environment, but others may be unculturable due to inefficient growth conditions (for the bacterium) being imposed upon them in the laboratory. There have been several recent reports regarding the physiological status of marine bacteria obtained from a variety of situations. Nilsson et al. (1991) reported that the estuarine bacterium *Vibrio vulnificus* became nonculturable within 27 days in nutrient limited conditions. Nilsson et al. (1991) also reported that there is a general trend amongst bacteria of a decreasing ability to be laboratory cultured from winter samples. This may be due in some part due to the transitory physiological status of the bacterium at the time of sampling, such as nutrient and light availability. For the enrichment programme samples were taken, where possible, during spring and summer to maximise the numbers of culturable bacteria within a sample. It was recognised that only a proportion of the bacteria in a sample might be

culturable, and only a portion of this population would be able to utilise MSA, so the size of the sample used was increased by tangential flow filtration.

Since many of the sample sites were coastal or close to estuary plumes, there was a possibility of isolating a freshwater organism. To reduce this possibility, samples were taken, where possible at high tide, to maximise the influx of seawater into the sample site. River systems can have a considerable influence on coastal seawater sites, e.g. the River Rhine can reduce overall salinity from 3.6‰ to 1.5‰ (w/v) NaCl upto 10 miles offshore (observation during Challenger Cruise 99A, Feb. 1993). This is a fairly extreme example given the volume of the Rhine, but demonstrates the effects rivers have.

The choice of carbon source used for enrichments was also carefully considered, since MSA-oxidizing bacteria were potentially only a small part of the total methylotrophic bacterial proportion. Most methylotrophic bacteria can utilise methanol, and many can utilise monomethylamine. By using these along with MSA as joint enrichment substrates it was hoped that enrichment of all methylotrophs, including MSA oxidizers, would occur. A similar philosophy was used when applying methane as an co-enrichment substrate. To obtain more diverse enrichment cultures, some short-chain alkylsulfonates were used (taurine,

cysteate, isethionate). In these cases, these were only used alongside MSA as an enrichment substrate. Longer chain sulfonates were not used as enrichment substrates so as to reduce the possibility of heterotrophic organisms being isolated. Several marine bacteria have been reported able to grow on linear-alkylbenzene sulfonates (LAS's), which are generally long chain residues from industry (Terzic et al., 1992). It is highly unlikely that such bacteria would be able to utilise MSA as a carbon source.

### **3.2. ISOLATION OF MARINE MSA OXIDIZING BACTERIA.**

Samples were taken from a variety of sites at various times of the year. Table 3.1. describes these sites at the time of sampling.

Over 80 separate enrichments were carried out, of which 20 yielded putative MSA oxidizing bacteria (representing all the sample sites) from the primary enrichment cultures. A very wide variety of enrichment conditions were employed including differing carbon source(s), growth temperature and the basal salts used. The microscopic observations on these enrichment cultures are listed in Table 3.2.

Site	Date(s)	Notes
Plymouth Sound	June 1992	Salinity ~3.2‰. Sample from surface taken 1 mile offshore
River Tamar	June 1992	Salinity 2.0-2.5‰. Sample taken at high tide.
North Sea	Aug. 1991	Sample taken by CTD* during research cruise
North Sea	Feb. 1993	Salinity 1.5-3.5‰. Various samples taken near the Rhine estuary in stormy conditions
Mid-Atlantic	July 1991	Sample taken by CTD* during research cruise
Celtic Sea	July 1992	Sample taken by CTD* during research cruise
Thorpe Bay, Essex	Dec. 1991	Sample taken from jetty at high water. Water was very turbid.
Wakering Steps, Essex	July 1992	Sample taken from juvenile salt marsh, at low water.
Penrith, Wales	July 1992	Sample taken from close to shore. Water quality was poor.
South Lynn, The Wash	Dec. 1991	Sample taken from irrigation ditch in well established salt marsh.

**Table 3.1. Marine sampling sites** . \*CTD (conductivity, temperature, depth) was a ship-board sampling device fitted with Niskin bottles and various sensory instruments (depth, salinity, pH, temperature, light, current speed and direction).

	Source	Cell Morphology	Enrichment method	Purity	Growth rate*
MA3	Mid Atlantic	<i>Spirillum</i> -like rods	a, b	>95%	14 d
MA4	Mid Atlantic	<i>Pseudomonas</i> -like rods	a, b	>70%	14 d
NA1	Mid Atlantic	Gram -ve small rods	a, c	>70%	5 d
NA5	Mid Atlantic	Gram -ve small rods	a, b	>70%	5 d
NS2	North Sea (Summer)	Gram -ve slender small rods	a, b	>95%	14 d
NS4	North Sea (Summer)	Gram -ve motile small rods	a, b	>70%	14 d
PS1	Plymouth Sound	Gram -ve short rods	a, b	>95%	7 d
PS2	Plymouth Sound	Gram -ve short rods	a, b	>95%	7 d
PS5	Plymouth Sound	Gram -ve short rods	a, b	>95%	7 d
PS6	Plymouth Sound	Gram -ve short rods	d, b	>70%	7 d
PS3L	Plymouth Sound	Gram -ve small rods	a, c	>70%	7 d
PS3V	Plymouth Sound	Gram -ve small rods	a, c	>95%	7 d
PSCH4	Plymouth Sound	Gram -ve thin rods	d, e	100%	13 d
PSMSA	Plymouth Sound	Gram -ve small rods	a, b	100%	60 d
TR2	Tamar Estuary	Gram -ve fat rods	d, b	>70%	7 d
TR3	Tamar Estuary	Gram -ve small thin rods	d, b	100%	7 d
WS2A	Wakering Steps	Gram -ve motile short rods	a, b	>95%	10 d
SL2	South Lynn	<i>Vibrio</i> -like rods	a, f	>70%	14 d
SL3	South Lynn	Gram -ve motile short rods	a, f	>70%	14 d
TE2	Thorpe Bay	Gram -ve long thin rods	a, b	>95%	7 d

**Table 3.2. Secondary enrichment cultures apparently growing using MSA as sole carbon and energy source.**

\*Growth "rates" in this case indicate time required after initial inoculation (starting OD<sub>600</sub> 0.05-0.06) before growth was visible (i.e. OD<sub>600</sub> was 0.1-0.2). Enrichment methods; a; conventional filtration, b; flask enrichment, c; agar tube culture, d; tangential flow filtration, e; chemostat enrichment, f; gradostat enrichment.

Three of these secondary enrichments were able to be further subcultured and successfully purified: PSCH4 (originally isolated from an MSA/methane fermenter enrichment, with water from Plymouth Sound as the inoculum), TR3 (isolated from 100x concentrated water from the River Tamar Estuary) and PSMSA (isolated from 100x concentrated water from Plymouth Sound). All three were obtained during the summer months. The salinity of this water was between 2.5 to 3.2% (w/v)

NaCl. Pure isolates were obtained from these 3 enrichment cultures by a rigorous programme of subculturing and plating on solid media. The enrichment cultures were unable to grow on agar- and silicon- based media, but did give discrete colonies on Phytagel-based media. Secondary enrichment cultures using the sloppy-agar-test tube technique (section 2.4.4.) did achieve some selectivity, by producing several discrete bands 5 to 10 cm below the surface; the mixed population was apparently separated by their respective affinities for oxygen. In several tubes containing Ellman's Reagent, a yellow pigmentation developed over time, apparently due to sulfite production. However, due to the diffusability of the agar used and the slow growth, it was not possible to localise this pigmentation to a particular band. In any case, colonies removed by Pasteur pipette could not be cultured further.

### **3.3. ISOLATION OF FRESHWATER MSA OXIDIZING BACTERIA.**

A wide variety of freshwater environments were sampled, which yielded a high number of enrichment cultures. Two cultures were selected for further study: FW2 (isolated from Tocil lake, University of Warwick campus) and FW6 (isolated from Finham Sewage Works, Warwickshire). These isolates were cultured on a simple ammonium mineral salts medium, MinE.



### 3.4. DISCUSSION.

Although a large number of enrichments (over 80) were carried out using marine samples, only 25% yielded a putative MSA oxidising culture. There was no absolute proof that all of these enrichments contained MSA-utilisers. An accurate method of determining MSA consumption (e.g. by Dionex ion-exchange chromatography, see appendix II) was not available at the time. Gas chromatography is sometimes used but is known to be very difficult, and the sensitivity of this method to small changes in MSA concentration is uncertain (S.F. Watts, personal communication). Sulfite production, indicated by incorporation of Ellman's Reagent into subculturing media, was assumed to indicate probable oxidative MSA consumption (though this would not indicate any alternative routes).

The fact that many of these enrichment cultures could not be further purified is typical of many marine bacteria, which are assumed to have specialist growth requirements. Only 4% of the enrichments could be successfully cultured and purified, indicating the difficulty of culturing marine bacteria, rather than perhaps the incidence of MSA-oxidizing bacteria in sea water. All sample sites showed some evidence of MSA-oxidizing bacteria. Few of the samples taken in winter yielded successful enrichments. This is probably due to the general culturing problems discussed by Nilsson *et al.* (1991). The only winter samples to yield

enrichment cultures were from the Wash (South Lynn) and the River Thames (Thorpe Bay, Wakering Steps). Both of these areas are subjected to high levels of certain nutrients, in particular nitrates and phosphates. The Wash receives a large load of mainly chemical fertiliser, from agricultural run-off. The Thames also receives, to a degree, a significant agricultural input. However, nitrates are particularly high, due to the activities of the water companies. The Thames is also the site of a large urban population, so it could be expected that there would a significant discharge of sewage. More isolates may be obtained by further sampling of seawater during the summer and spring months.

There may have been a degree of selectivity in the enrichment procedure. A substantial portion of the bacterial population may have been excluded by the prefiltration step. It is thought that particulate matter may offer microhabitats to bacterial assemblages (DeLong et al., 1994), either as transitory microaerophilic or anaerobic environments. Since this particulate matter (marine snow, general detritus) was removed by filtration, to prevent heterotrophic growth, an unknown portion of the population was excluded from enrichment. It is possible that by including this fraction of the bacterial population, the variety of isolates obtained could have been increased. A further degree of selectivity was produced by the inclusion of 3.0%

(w/v) sodium chloride into the media used, i.e. the average NaCl content of sea water. Most bacteria are in some way salt-sensitive. Strain M2 did not grow at concentrations above 0.5% (w/v) NaCl (Baker et al., 1991), so it was considered unlikely that terrestrial or freshwater MSA oxidizing bacteria would be inadvertently isolated at elevated NaCl concentrations. Indeed, many marine bacteria have an obligate requirement for sodium ions.

Methylotrophic substrates, besides MSA, were included in the media to enable the methylotrophic bacterial population to out-compete heterotrophic bacteria. Most methylotrophic bacteria are known to be able to utilise methanol, and some can utilise monomethylamine. This approach was more successful than by using MSA alone as the carbon source. Similar findings were reported by Baker (1992) when enriching for soil MSA oxidizing bacteria. However the reason for this is not clear. Methylotrophic bacteria were readily enriched from all seawater samples, using methanol as a carbon source, so the reason is clearly not related to the general physiology of marine methylotrophic bacteria *per se*. Given that successful MSA enrichments were obtained from only summer samples, it may well be that the culturability of marine MSA-oxidizing bacteria is "event-linked"; during the spring and summer months, DMS concentrations increase as a result of seasonal phytoplankton blooms, resulting in an increase in the

aerosol MSA concentration. MSA is returned to seawater intermittently by wet deposition. MSA concentrations may only be sufficient to induce the necessary enzymes for a short period (during blooms). A more likely explanation is shown by a small experiment using the tangential flow filtration apparatus. Two filters were available, 0.3  $\mu\text{m}$  and 1000 kDa. Using methanol as a carbon source, enrichment cultures were obtained from the filtrates (i.e. "cell-free") of two identical seawater samples (Plymouth Sound, sampled in summer) concentrated as described. Growth was found only in the 0.3  $\mu\text{m}$ -filtered seawater enrichments, indicating that methylotrophic bacteria smaller than 0.3  $\mu\text{m}$  were passing through the filter. The inference from this is many potential MSA oxidizers were lost through the filtering technique adopted for early enrichments. Microscopic examination of these cultures showed cells of approximate size 3 by 0.5-1.0  $\mu\text{m}$ . It is not known what effect this apparent size difference of cells in culture from those in natural environments has on the biochemistry and growth characteristics of bacteria.

There are several reports of marine methanotrophic organisms being unable to be cultured on conventional solid media (Lees et al, 1991, Distel and Cavanaugh, 1994). There seem to be few recent reports of new *bona fide* marine facultative methylotrophs, perhaps because of the cultivation problems and the uncertainty about contamination. All three marine strains isolated grew on Phytigel, rather than agar, the reason behind this

is uncertain. It seems unlikely that these marine bacteria are unable to grow on agar because of the use of agar *per se*. Agar has been used successfully for the enrichment of many types of bacteria. However, it is known that marine bacteria are sensitive to high concentrations of certain substances, possibly due to the oligotrophic nature of their natural habitat. Clearly, in the case of these strains this appears to be selective, high concentrations of substrate, salts and trace elements are tolerated, and indeed promote growth. These additions are not comprehensive however; there may be certain key trace compounds present in agar preparations not normally found in media which the bacteria are sensitive to. There is clearly a dilution effect since enrichments were obtained in "sloppy-agar" tube enrichments. This trace substance may be introduced during the commercial refining of agar, or it may be a natural constituent of agar. Improved recognition of bacterial growth requirements may improve enrichment procedures with regard to marine bacteria.

There have been several reports indicating that MSA can be used as a sulfur source by several soil and enteric bacteria (Cook and Hütter, 1982, Uria-Nickelson et al., 1993, Chien et al., 1995). In these cases, MSA does not appear to be utilised as a carbon source, probably because the bacteria concerned do not possess the necessary enzymes for the assimilation of

C<sub>1</sub> compounds. This feature of bacterial physiology was not examined in this research.

### 3.5. CONCLUSIONS.

Future enrichment and isolation of novel marine MSA-utilising bacteria will be improved with regard to the general trends found during this study. Judicious use of co-enrichment substrates may increase the diversity of isolates, and lead to the isolation of novel microorganisms. It seems clear, for seawater obtained from around the British Isles at least, that limited results will be obtained from using winter seawater samples. The use of agar-based media should be treated cautiously; it seems likely that its use will underestimate the diversity of marine bacteria. Similarly, enumeration of marine bacteria by total-count methods (using agar plates) will also produce misleading results. A method for isolating new organisms on solid media that may be worthy of further investigation is the use of tube-cultures (producing a gradient of oxygen tension), either using agar or Phytigel. Clearly, a proportion of the bacterial population in surface waters is microaerophilic, hence it is unlikely that these would be detected on conventional plates under a normal oxygen tension.

## CHAPTER 4: PHYSIOLOGICAL CHARACTERISATION OF MARINE AND FRESHWATER ISOLATES.

### 4.1. INTRODUCTION.

Physiological characterisation of bacteria here refers to a classical microbiological study of the isolates TR3, PSCH4, FW2 and FW6. Although such studies are of limited use with reference to the taxonomy of these methylotrophic organisms, they are of importance in establishing ideal cultivation conditions, differences between the isolates, and, for the marine strains, establishing the legitimacy of the claim that such isolates are *bone fide* marine methylotrophic bacteria. Few such bacteria have been isolated from marine sources. Those that have, are mostly restricted or obligately facultative methylotrophs (Green, 1992). High magnification microscopy can reveal structures (internal membranes, the presence of extracellular slime-like matrices) which may explain certain morphological and biochemical behaviours. A study of physiology would also allow improved enrichment strategies to obtain further novel isolates.

A full characterisation in the sense of that applied to many medical bacteria was not applied here, mainly due to the limited information that such exhaustive tests would reveal, but also many methylotrophic bacteria would produce misleading results in such tests with many false negatives. Most of these tests

(i.e. fermentation tests,  $\beta$ -galactosidase, H<sub>2</sub>S formation) are based around complex media, which are not conducive to growth for many methylotrophs (Baker, 1992). Lidstrom (1992) and Green (1992, 1993) described the general characteristics that distinguish methylotrophic bacteria from other bacteria. There appears to be few distinguishing features, taxonomic determination appears to be mainly on the basis of mode of metabolism and pigmentation produced. In this respect, the traditional taxonomy of methylotrophic bacteria is limited. The most useful taxonomic information would probably be gleaned from molecular characterisation, such as 16S rRNA gene sequencing or fatty acid profiling.

#### **4.2. INITIAL DESCRIPTION OF MARINE STRAINS TR3 AND PSCH4.**

Marine strains TR3, PSCH4 and PSMSA were isolated from sea water and grew using MSA as a sole carbon source. All three isolates were gram negative non-spore forming rods. Due to their unusual, and distinctive, morphology, TR3 and PSCH4 were selected for further study. TR3 and PSCH4 were morphologically very similar, with a cell size of around 2 x 0.3 to 0.5  $\mu$ m (determined from scanning electron microscopy). Both had a tendency in liquid culture, from early in the exponential growth phase to form distinctive finger-like clumps of cells (Fig. 4.1. to 4.6.) different from those reported by Whittenbury et al. (1970). This



is a trait that has not been reported in  
methylophilic bacteria.

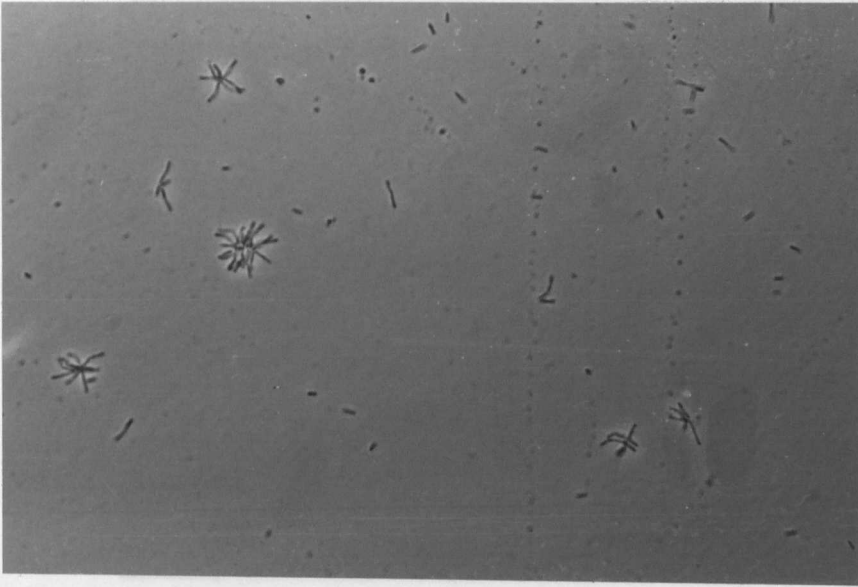


Fig. 4.1. Scanning electron micrograph of strain H2.

Fig 4.1. Light micrograph of strain TR3 (x1000).

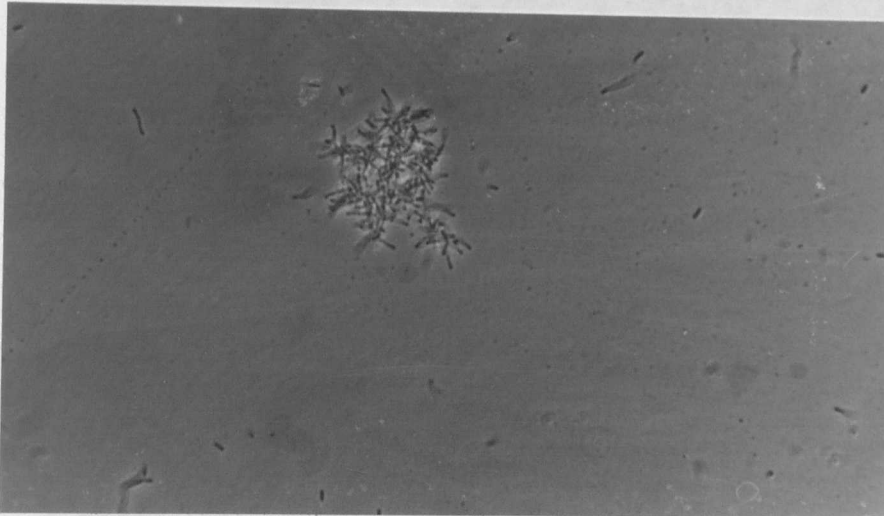


Fig. 4.2. Light micrograph of strain PSCH4 (x1000).

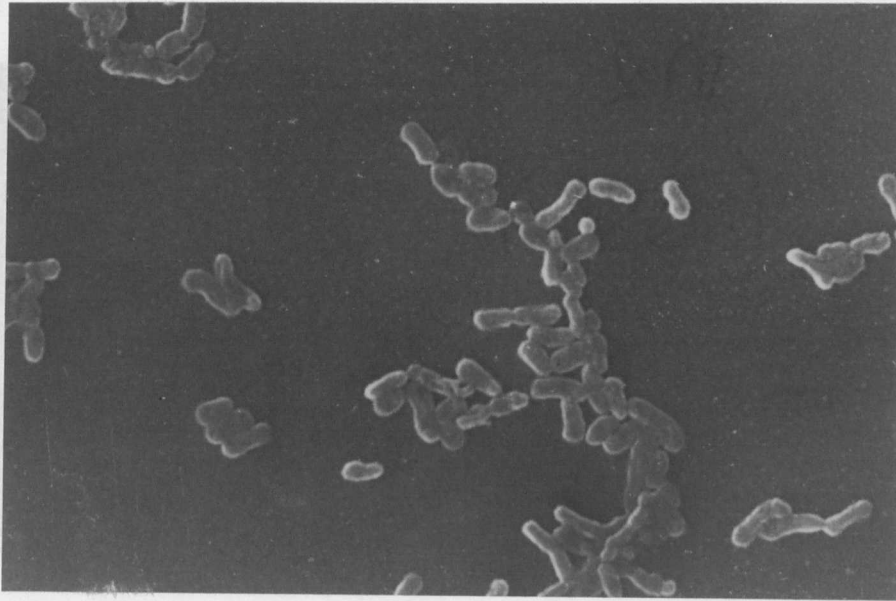


Fig. 4.3. Scanning electron micrograph of strain M2  
(x3500)

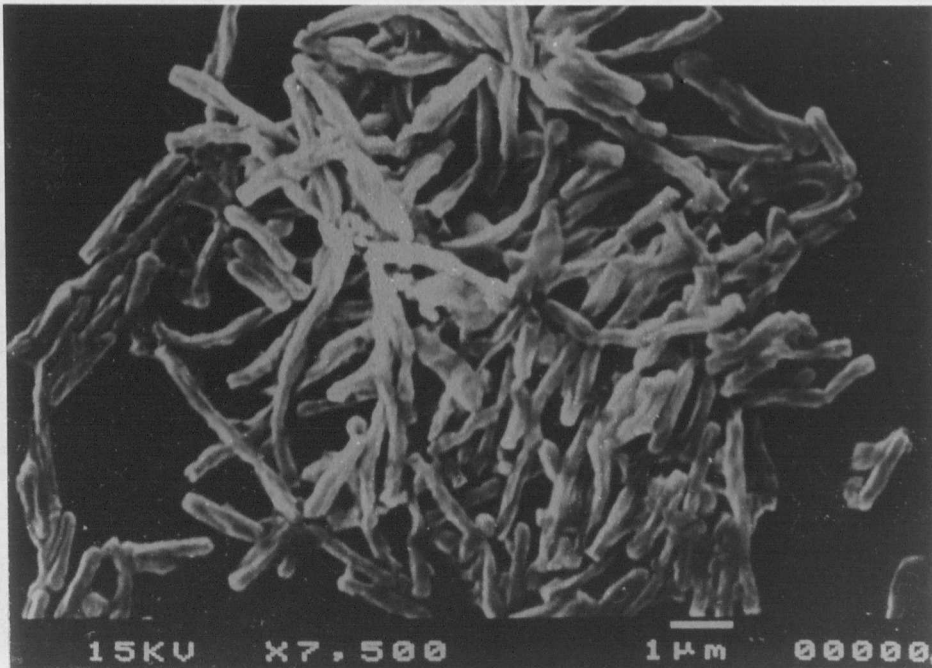


Fig. 4.4. Scanning electron micrograph of strain PSCH4  
(x7500)



Fig. 4.5. Scanning electron micrograph of strain TR3 (x5000).

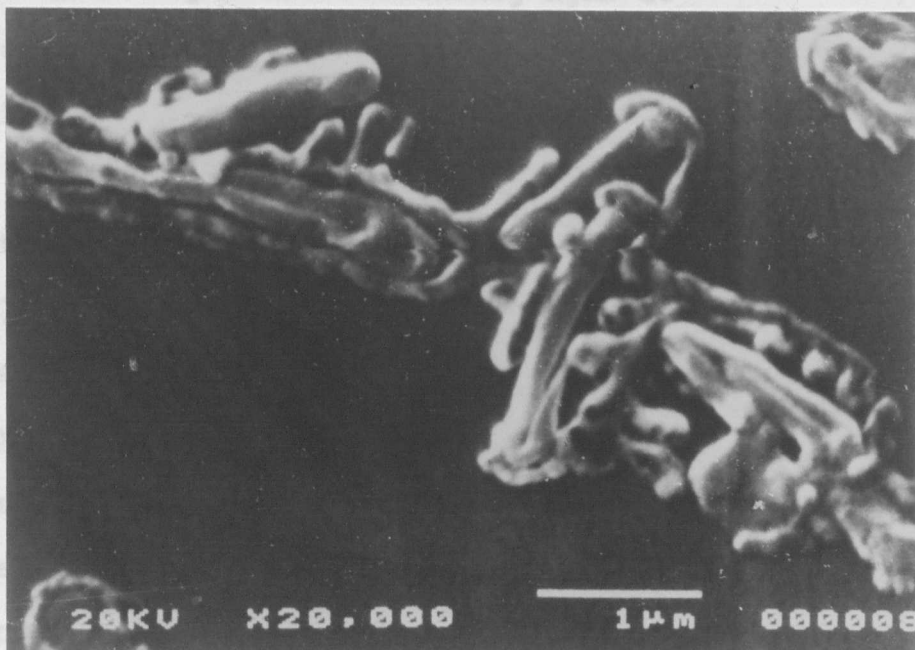


Fig 4.6. Scanning electron micrograph of strain TR3, showing slime-like matrix (same field of view as Fig. 4.5.) (x20000).

Careful examination of scanning electron micrographs (at x 20000) showed the presence of a gelatinous matrix, into which the cells were embedded. Neither strain possessed a capsule and both strains possessed cytochrome oxidase. Although both strains gave a positive reaction with the test for oxidase, strain TR3 was only weakly positive. Neither strain grew readily on solid surfaces, with little growth observed on glass surfaces (i.e. on the inside of glass flasks) or grew in nutrient broth, either at standard NaCl concentrations or at 3.0% (w/v) NaCl. Growth was not possible in any media containing agar at concentrations of above 0.5% (w/v), or silica plates (Table 4.1.).

Growth was possible in media containing up to 1.0% (w/v) Phytigel (or Gelrite), which gave very small raised, colourless colonies (diameter less than 1 mm) after 10 days incubation. These colonies could be successfully transferred to liquid culture, to give the same distinctive rosettes observed previously. Although growth was observed at temperatures down to 20°C, the optimal growth temperature was found to be around 30°C, with poor growth at 37°C.

Although a vitamin supplement was not required for growth, a pronounced growth lag was observed for both strains in the absence of such a supplement and a comprehensive trace metal supplement was required for both strains. Use of a simpler trace metal solution

(Lees et al., 1991) resulted in qualitatively poorer growth compared to using the trace metal supplement defined by Whittenbury et al. (1970).

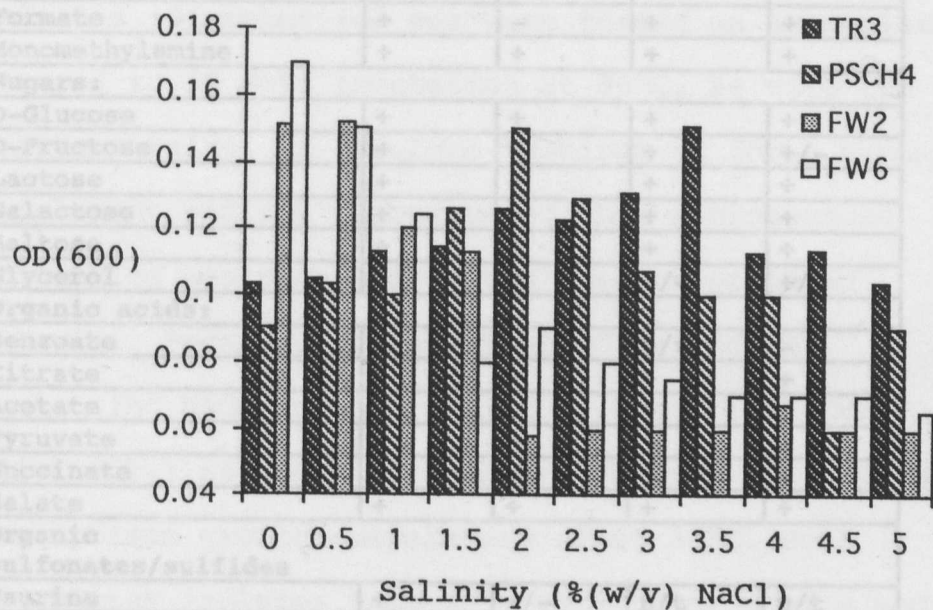
Characteristic	Strain TR3	Strain PSCH4
Gram reaction	negative	negative
Capsule stain	negative	negative
Cell morphology	elongated rods	elongated rods
Cell size	2 x 0.3 $\mu\text{m}$	2 x 0.5 $\mu\text{m}$
Optimum growth temperature	30°C	30°C
Doubling time on MSA (h)	7.0	7.2
Growth at 37°C	poor	poor
Optimum salinity (NaCl % (w/v))	2.0-3.5	1.5-2.5
Salinity growth range (NaCl % (w/v) )	0.5-4.5	0.5-3.5
Growth on agar over 10 days*	no	no
Growth on Phytigel over 10 days <sup>+</sup>	yes	yes
Catalase test	positive	positive
Oxidase test	weakly positive	positive
Mol% G+C content of DNA	57.0	56.8

**Table 4.1. Physiological characteristics of marine strains TR3 and PSCH4.** Notes. \*; growth tested in range 1.5-2.0% (w/v) agar. +; growth tested in range 0.5-1.0% (w/v).

The optimum salinity observed (determined from optical density observed after 7 days growth) for strain TR3 was in the range 2.0-3.5% (w/v) added NaCl



and for strain PSCH4 in the range 1.5–2.5% (w/v) added NaCl (Fig. 4.7.).



Fi

Figure 4.7. Effect of salinity on growth after 7 days of strains TR3, PSCH4, FW2 and FW6. OD(600) was measured

after 7 days incubation at 30°C, with 25 mM MMA as carbon source. OD(600) at T=0 was between 0.05 and 0.06 units for all PSCH4, FW2 and FW6. +) (OD<sub>600</sub> > 0.2) growth (after 7 days),

+/-) (0.06 < OD<sub>600</sub> < 0.20 post growth (after 7 days), -) (OD<sub>600</sub>

< 0.06) no growth (after 14 days incubation). n/b: not tested.

Both strains failed to grow at concentrations above

5.0% (w/v) NaCl, but showed growth at NaCl

concentrations as low as 0.5% (w/v). Strains TR3 and

PSCH4 were both facultative methylotrophic bacteria,

since they grew on a wide range of sugars, organic

acids and C<sub>1</sub> compounds as a sole source of carbon and

energy. Neither strain TR3 and PSCH4 could utilise

glycerol, and could only utilise a limited range of

sulfonated compounds other than MSA. The range of

substrates tested is shown in Table 4.2.

Substrate	Strain TR3	Strain PSCH4	Strain FW2	Strain FW6
<b>C<sub>1</sub> substrates:</b>				
MSA	+	+	+	+
Methanol	+	+	+	+
Formaldehyde	+	-	+	+
Formate	+	-	+	+
Monomethylamine	+	+	+	+
<b>Sugars:</b>				
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+/-
Lactose	+	+	+	+
Galactose	+	+	+	+
Maltose	+	+	+	+
Glycerol	-	-	+/-	+/-
<b>Organic acids:</b>				
Benzoate	-	-	n/t	-
Citrate	+	+	+	+
Acetate	+	+	+	+
Pyruvate	+	+	+	+
Succinate	+	+	+	+
Malate	+	+	+	+
<b>Organic sulfonates/sulfides</b>				
Taurine	+	+/-	n/t	n/t
Isethionate	+	+	n/t	n/t
Cysteate	-	-	n/t	n/t
Ethanesulfonate	-	-	n/t	n/t
Dimethylsulfide	-	-	n/t	n/t

**Table 4.2. Substrate growth profiles of strains TR3, PSCH4, FW2 and FW6.** +; (OD<sub>600</sub> > 0.2) growth (after 7 days), +/-; (0.06 < OD<sub>600</sub> < 0.20 poor growth (after 7 days), -; (OD<sub>600</sub> < 0.06) no growth (after 14 days incubation). n/t; not tested.

#### **4.3. Characterisation of freshwater strains FW2 and FW6.**

Strains FW2 and FW6 both utilised MSA as their sole source of carbon and energy. Both strains were gram-negative, non-spore forming rods of approximately 3 x 0.5  $\mu\text{m}$  (Table 4.3.).



Morphologically both strains were very similar forming short fat rods and did not form clumps, as had been observed with strains TR3 and PSCH4. Discrete white colonies (diameter 1-2 mm) were formed on agar plates after 7 to 10 days incubation at 30°C. As with the marine strains, an absolute requirement for a vitamin supplement was not demonstrated, but a pronounced growth lag was observed in the absence of added vitamins. Poor growth was observed in medium containing NaCl concentrations of above 1.0% (w/v) (Fig. 4.7.) and both strains grew poorly at 37°C, with the optimum growth temperature about 30°C. Both freshwater isolates grew on a variety of sugars and organic acids (Table 4.2.) as sole carbon and energy sources. FW6 was unable to utilise benzoate (20 mM) (FW2 was not tested). Growth on glycerol was poor. FW6 showed poor growth on fructose. Neither strain grew in a nutrient broth medium.

	Strain FW2	Strain FW6
Gram reaction	negative	negative
Capsule stain	negative	negative
Cell morphology	motile single small rods	motile single small rods
Cell size	2.5 x 0.5 $\mu\text{m}$	3.5 x 0.5 $\mu\text{m}$
Doubling time on MSA (h)	8	8.5
Growth at 37°C	poor	poor
Optimum salinity (NaCl % (w/v))	< 0.5	< 0.5
Salinity growth range (NaCl % (w/v))	0-2.0	0-2.0
Growth on agar	Yes	Yes
Catalase test	positive	positive

**Table 4.3. Physiological observations of freshwater strains FW2 and FW6.**

#### **4.4. GROWTH OF OTHER BACTERIA ON MSA.**

Initial examination of the 16S rRNA gene sequence of strain TR3 by Dr. A. Holmes (University of Warwick, see Appendix I) suggested there was significant homology with *Pseudomonas diminuta*, *Paracoccus denitrificans* and *Rhodopseudomonas sphaeroides*. Type strains of these organisms were obtained from the NCIMB and inoculated into the appropriate medium, with MSA as the carbon source. No growth was observed on MSA in any of the strains tested.

#### 4.5. GROWTH REQUIREMENTS OF STRAINS PSCH4 AND TR3.

##### 4.5.1. Preferred nitrogen source.

For all strains (freshwater and marine), nitrate appeared to be the preferred nitrogen source (Table 4.4.), when compared to other possible nitrogen sources (ammonium ions, gaseous nitrogen (under a range of oxygen tensions)). However, it should be noted that the apparent reduced requirement for ammonium ions may be due to changes in pH of the medium.

Nitrogen Source	FW2	FW6	TR3	PSCH4
No carbon source <sup>a</sup>	0.062	0.066	0.089	0.092
No N-source	0.065	0.063	0.090	0.095
NO <sub>3</sub> <sup>-</sup>	0.158	0.162	0.143	0.139
NH <sub>4</sub> <sup>+</sup>	0.099	0.110	0.113	0.120
N <sub>2</sub> : 100% <sup>b</sup>	0.056	0.055	0.085	0.086
N <sub>2</sub> : 90% <sup>b</sup>	0.068	0.064	0.085	0.085
N <sub>2</sub> : 85% <sup>b</sup>	0.065	0.067	0.084	0.087
N <sub>2</sub> : 80% <sup>b</sup>	0.061	0.062	0.089	0.090

**Table 4.4. Preferred nitrogen source for strains FW2, FW6, TR3 and PSCH4.** Relative growth using different

nitrogen sources reflected as the optical density of similarly inoculated cultures (with MSA as the carbon source) measured at 600 nm after 7 days. <sup>a</sup>Cultures with no added carbon source used MAMS as basal salts medium. <sup>b</sup>Gaseous nitrogen was added to MS medium lacking an added nitrogen source by sparging sealed flasks with oxygen-free nitrogen.

If nitrogenase (oxygen sensitive) was present, one would expect some sort of variation in growth with oxygen concentration. Neither marine strain showed a requirement for reduced levels of oxygen. This does not conclusively demonstrate the absence of nitrogenase since the cells would be expected to have considerable reserves of nitrogen or be able to scavenge trace amounts of nitrogen in the medium. To demonstrate nitrogen fixation conclusively, one would need to measure  $N_2 \rightarrow NH_3$  (through use of  $^{15}N$ -labelled substrates) or carry out the acetylene reduction assay. Although the growth level (i.e. optical density at 600nm) of the marine strains was higher after 7 days than that obtained with the freshwater strains, growth in the absence of added nitrogen source was comparable to that found with no added carbon source. This difference in growth between strains can be explained by inconsistencies of the inocula used.

#### **4.5.2. Growth on solid media.**

Neither marine strain grew successfully on any agar-based solid media a full range of suitable solidifying agents was tested at various concentrations (Table 4.5.). Using agar at a concentration of 0.4% (w/v) gave a semi-fluid matrix, which appeared to allow limited growth of colonies, when inoculated as a pour-plate. However, this was inconvenient for routine work, and was unsuitable for short term storage. Similarly, silica-based solidifying agents also failed

to allow growth. Gelrite (Merck) and Phytigel (Sigma), two similar Gellan gum based solidifying agents (used routinely for plant tissue culture) gave similar small colonies after 7-9 days incubation with both marine strains, which could be successfully subcultured into liquid media.

Solidifying Agent	Strain TR3	Strain PSCH4
Bactoagar, 2.0% (w/v)	-	-
Bactoagar, 0.4% (w/v)	+	+
Noble Agar, 2.0%(w/v)	-	-
Noble Agar, 0.4%(w/v)	+	+
Silica Gel, 0.5%(w/v)	-	-
Ludox	-	-
Phytigel, 0.5%(w/v)	+	+
Phytigel, 1.0%(w/v)	+	+
Gelrite, 0.5%(w/v)	+	+

**Table 4.5. Effect of solidifying agent on the growth of marine strains TR3 and PSCH4.** Each solidifying agent was added to MAMS, with MSA as the carbon source. Growth was taken as the production of colonies which could then be subcultured into liquid media. If growth occurred on plates, but subculturing into liquid medium was unsuccessful, such growth was assumed to be contamination by non marine bacteria. Plates were incubated at 30°C for up to 14 days. Colonies were screened by using a plate microscope.

Microscopic observation of colonies growing on Phytigel and Gelrite showed the same distinctive cell types as that found in liquid culture. Few contaminating organisms appeared to grow on plates

containing the Gellan gum, allowing a convenient method to purify contaminated cultures. Cyclohexamide (10 mg/l) could be successfully incorporated into Phytigel plates to reduce fungal contamination.

#### **4.6. THE MOL% G+C CONTENT OF DNA EXTRACTED FROM STRAINS TR3 AND PSCH4.**

The mol% G+C content of DNA determined for strains TR3 and PSCH4 were 57.0 and 56.8 respectively. The values determined for the controls were as follows (published values from Brock and Madigan, 1991); *Micrococcus luteus*, 67.6 (65-75), *Clostridium perfringens*, 26.2 (24-27), *Escherichia coli*, 52 (50-51). Contrary to that reported for strain M2 (Baker, 1992), the caesium chloride gradient centrifugation method used here was successful in obtaining DNA of high enough purity for the determination of mol% G+C content.

#### **4.7. DISCUSSION.**

All four isolates studied in some detail shared the common traits of being gram-negative facultative methylotrophic bacteria. Morphologically, the marine isolates differed from the freshwater strains, and showed a requirement for salt (as NaCl) at approximately the concentration found in seawater. Previous reports describing optimum salt growth concentrations for marine methylotrophic organisms are somewhat variable, from around 0.4% (w/v) NaCl (Strand

and Lidstrom, 1984) to 3.0% (w/v) NaCl reported for *Methylophaga marina* (Urakami and Komagata 1987). The wide salinity growth ranges reported here may be attributable to the tidal nature of the sites the isolates were obtained from. The Tamar Estuary (and to a lesser extent, Plymouth Sound) is subject to daily large fluctuations in salinity during tidal changes (from approximately 0.5% (w/v) NaCl at low water to 3.5% (w/v) at high water).

Few deductions regarding the phylogenetic positioning of any of the isolates from the initial phenotypic description can be made, as there appears to be little in common between facultative methylotrophs except their mode of metabolism (Green, 1993). However, the traits reported here are generally typical of the group *Methylobacterium*. Since neither marine strain, nor FW6 (FW2 not tested) showed an ability to grow using benzoate as a carbon source would seem to indicate that these strains were not *Pseudomonas fluorescens*-like bacteria (Brock and Madigan, 1991).

The mol% G+C DNA contents of strains TR3 and PSCH4 (57.0 and 56.8 respectively) are similar to that found for the strain M2 (61 (Kelly et al., 1994)). These values are lower than that found for *Methylobacterium* (68.4 to 72.4 mol% G+C, Green, 1992). In themselves, the mol% G+C content values do not necessarily suggest relatedness; gram positive and gram negative bacteria can have very similar values.

All four strains are fairly typical facultative aerobic methylotrophs, being able to grow on a wide range of substrates. The inability of strain FW6 to grow on fructose is by no means inconsistent with the likelihood of all four strains being grouped into the same genus, the group *Methylobacterium* shows such a pattern (Green, 1993). The degree of caution towards conventional physiological characterisation (i.e. using API strips) was justified by the inability of any of the isolates to grow in a complex liquid medium (nutrient broth). API tests with the strain M2 gave a false identification of this isolate as *Pseudomonas paucimobilis*, based upon a large number of the tests conducted being negative. It was concluded that this was probably due to the presence of a standard complex medium base in most of the tests (Baker, 1992). The same appears likely with the new isolates.

The only marine facultative methylotrophs reported are *Methylophaga marinus*, *Methylophaga thalassica* (Janvier et al., 1985), *Altermonas thalassomethanolica* and *Methylomonas thalassica* (Yamamoto et al., 1980). These would be better defined as restricted facultative methylotrophs, since they could only utilise a narrow range of sugars, and are, hence, unlikely to be particularly closely related to the new strains described here. The phylogeny of the MSA-oxidizing isolates obtained in this study (TR3, PSCH4, FW2 and FW6) can only be fully resolved with a full analysis



of the 16S rRNA gene sequence of these strains (see Appendix I).

All four strains indicated a preference for nitrate as a nitrogen source (Table 4.4.), though there was some evidence of ammonium utilisation. This is not inconsistent with known bacterial nitrate assimilation pathways. There was no evidence of nitrogen fixation, though this feature appears to be variable amongst methylotrophic bacteria (Green, 1992).

The peculiar growth characteristics of strains TR3 and PSCH4 on solid media have been reported for two marine methanotrophic strains isolated from the same area (Lees et al., 1991), but not for the marine facultative methylotrophs reported (Janvier et al., 1985). It is unlikely that the cause of this lack of growth would have been due to agar itself, but more likely that it was due to the presence of some growth inhibitor in the agar preparation, which was subsequently diluted sufficiently at low concentrations to allow some growth. Gellan gum is derived from a polysaccharide produced by *Pseudomonas elodea* (Harris, 1985) and supplied, in a highly purified form, as suitable for plant tissue culture. It is unlikely that there is a contaminant growth factor within the preparation, or that the polymer provides some sort of substrate for the bacteria, as there was no indentation in the plates from colony growth. Without a complete chemical analysis of agar

preparations, however, it would be impossible to speculate further. There are several reported incidences of Phytigel or Gelrite-based media providing an improved growth medium for the cultivation of other bacterial species (Shungu et al, 1983, Lin and Casida Jr., 1984, Harris, 1985). Gellan gum based media generally provides a medium that gives higher total counts, has excellent clarity and good water retention (useful for both slow growing and thermophillic micro-organisms).

#### 4.8. CONCLUSIONS.

Marine strains TR3 and PSCH4 are facultative MSA-utilizing methylotrophic organisms, typical of the *Methylobacterium* genus. They show peculiar morphological characteristics, growth characteristics on solid media and a requirement for added sodium chloride that distinguish them from strain M2 and other facultative bacteria previously described. In other respects, their physiology is very similar to that reported for strain M2. The mol% G+C of both strains is sufficiently different from that reported for strain M2 (Kelly et al., 1994) for them to be judged new species, but of the same genera. The freshwater strains FW2 and FW6 also appear to be facultative methylotrophs, with characteristics very similar to those reported for M2 (Kelly et al.1994).

## CHAPTER 5: PHYSIOLOGICAL AND BIOCHEMICAL STUDIES ON MARINE ISOLATES TR3 AND PSCH4.

### 5.1. INTRODUCTION.

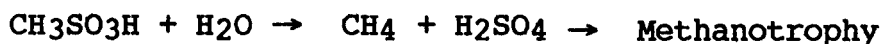
The characterisation of some key biochemical pathways in the marine strains TR3 and PSCH4 was carried out using the whole-cell and cell-free techniques described in sections 2.7. and 2.8. Most previous studies have investigated the utilisation of low molecular weight sulfonates as sulfur sources (e.g. Thyse and Wanders, 1972, Uria-Nickelsen et al., 1993). One report described the utilisation of MSA as a carbon source by a soil bacterium, strain M2 (Kelly et al., 1994), by following a whole-cell and cell-free approach. By analysing patterns of oxidation of certain C<sub>1</sub> compounds *in vivo* and by assaying for certain key enzymes in methylotrophic biochemistry, it was possible to deduce the likely pathway of MSA oxidation in both marine strains.

To determine the route of MSA metabolism in strains TR3 and PSCH4, one needs to be aware of the various possible routes of MSA utilization:

#### A. Methanogenesis.

Here MSA is either anaerobically desulfonated to methane, or is used as a sulfur source in aerobic bacteria, whereby methane would be generated as a side

product. Clearly, strains TR3 and PSCH4 are not anaerobes, so aerobic methanogenesis must be considered. There have been several reports of MSA being used as a sulfur source by certain species of *Pseudomonas* and enteric bacteria (Cook and Hütter, 1982, Seitz et al., 1993). The metabolism of MSA *per se* in these cases was not studied in detail, but one can assume that as these bacteria are all heterotrophs, then methane was not utilised. For this pathway to be a real possibility in the case of the utilisation of MSA as sole carbon and energy source for an aerobic bacterium, methane oxidation must be demonstrated. Sulfur in this case would be released as sulfate, i.e.:



#### B. Methyilotrophic pathway via methanol.

Given that in MSA, the weakest bond is the C-S bond, it is quite conceivable that this would be the site of initial enzymatic cleavage, releasing a methyl group which would hydrolyse to methanol. Further oxidation would then proceed via the methyilotrophic pathways discussed in section 1.5. Demonstration of this pathway would be difficult. If methanol is subsequently oxidized to formaldehyde, one would have to demonstrate that inhibition of methanol oxidation somehow affects MSA metabolism. Use of an MDH inhibitor would affect the oxygen: substrate

stoichiometry for MSA. Methanol should also be detected in culture media. For definitive proof of this pathway, MDH<sup>-</sup> mutants would need to be produced, to demonstrate the effect on MSA metabolism. Sulfite would be the probable sulfur-moiety metabolite.

#### C. Methylotrophic pathway via formaldehyde.

MSA may be oxidized directly to formaldehyde, with the cocomitant release of sulfite. Methanol would not be an intermediate. The existence of this pathway would be shown by the same studies described in 5.1.2. If inhibition of MDH does not affect MSA metabolism, then methanol is unlikely to be an intermediate.

#### D. Oxidation of MSA via a carrier molecule system.

In *Pseudomonas* MS (Kung and Wagner, 1970), oxidation of methyl-group compounds proceeds via a carrier molecule, such as tetrahydrofolate. In the case of *Pseudomonas* MS, methyl containing groups (e.g. trimethyl sulfonium) are transferred to the carrier molecule (i.e. tetra-hydrofolate). The methyl group is oxidized as a side chain of the carrier molecule, and released as formate. Such a system would be difficult to demonstrate by conventional enzyme assaying, except that the lack of certain methyl-otrophic enzymes could indicate the presence of such a system. The oxygen: substrate stoichiometry would also be affected.

Neither strain utilised methane as a carbon substrate, so the methanogenic route is unlikely. However, it may be possible to obtain apparent growth on MSA, if the organisms are autotrophic. If so, both strains would probably utilise the ribulose biphosphate pathway, and so possess ribulose-biphosphate carboxylase (RuBISCO). Oxygen: substrate stoichiometry patterns would indicate the absence or presence of a carrier molecule system.

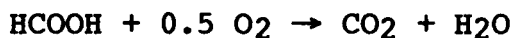
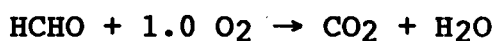
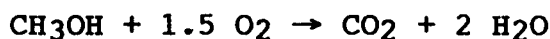
The pathway of MSA oxidation by strains TR3 and PSCH4 was determined by a combination of whole cell and cell-free extract assaying. Analysis of culture supernatants was also carried out to investigate the presence or absence of likely intermediates.

## **5.2. WHOLE-CELL STUDIES OF ISOLATES TR3 AND PSCH4.**

### **5.2.1. Oxygen : substrate ratio studies.**

Table 5.1. shows the oxygen-substrate stoichiometries determined for a range of key C<sub>1</sub> compounds, tested with strains TR3, PSCH4, FW2 and FW6 grown on MSA.

These data indicated the number of moles of oxygen required for the complete oxidation of a range of carbon sources to carbon dioxide. The stoichiometries obtained for both strains were consistent with the following series of reactions:



Substrate	TR3	PSCH4	FW2	FW6
MSA	2.07 (16)	2.08 (15)	1.9 (4)	2.07 (4)
MSA + 10mM Cyclopropanol	1.95 (3)	1.98 (3)	n/t	n/t
Methanol	1.45 (4)	1.44 (4)	1.46 (4)	1.36 (3)
Methanol + 10mM Cyclopropanol	0	0	n/t	n/t
Formaldehyde	1.2 (3)	1.1 (3)	n/t	n/t
Formate	0.51 (4)	0.56 (5)	0.60 (3)	0.60 (3)

**Table 5.1. Oxygen: substrate ratios of various substrates tested with cell suspensions of strains TR3 and PSCH4.** Numbers in parenthesis indicate number of separate determinations. Values quoted are means of these. n/t; not tested

Dimethyl sulfide and methane were also tested, but were not oxidized by any of the strains tested. Addition of 10 mM cyclopropanol, a potent inhibitor of pyrroloquinoline quinone (PQQ)-linked enzymes (Frank *et al.*, 1989), did not affect the stoichiometry obtained with MSA, but completely inhibited methanol oxidation by MSA-grown cells. From this, it may be concluded that a PQQ-linked enzyme was not involved directly in the oxidation of MSA, although a PQQ-linked methanol dehydrogenase was expressed in MSA-grown cells.

MSA-grown cells showed a consistently high endogenous rate of oxygen consumption, even after the rigorous washing programme described in section 2.7.5.2. With a high endogenous oxidation rate it was difficult to determine consistent oxygen consumption data. By incubating cells at 30°C for 2-3 hours prior to testing, consistent stoichiometries could be estimated. The high endogenous oxygen consumption rate may have been due to the rosettes previously reported in Chapter 4. The incubation period may have allowed exopolysaccharides (causing cells to clump together) to be metabolised, thus reducing the endogenous oxidation rate. Since this clumping was found in both flask-grown and fermenter-grown cells, this is unlikely to be a result of pH changes in culture. Oxidation of methanol by either of the marine strains, TR3 and PSCH4, was also difficult to demonstrate. Initially methanol was not oxidized by MSA-grown cells of both marine strains, but by freeze-thawing the cells and applying one washing and centrifugation step, it was found the cells oxidized methanol with only a short lag period (10-20 s). This may have been due to the presence of the polysaccharide. By breaking up the clumps (by freeze-thawing) methanol may have been more accessible to the cells. No other investigations to see if other methods would also work (e.g. mild sonication) were undertaken, since the initial problem seemed to have been solved.



### 5.2.2. Substrate oxidation specificity of marine strains TR3 and PSCH4.

Table 5.2. illustrates the relative oxidation rates determined for a range of substrates, using the oxygen electrode and MSA-grown cells (marine strains TR3 and PSCH4), with a substrate concentration of 1.7 mM. A range of C<sub>1</sub> substrates and alkyl sulfonates was also tested.

Methanol was found to be oxidized at a slower rate, compared to MSA. Conversely formate and formaldehyde were both oxidized more quickly in the case of strain TR3. For strain PSCH4 the rates were 75% and 55% respectively of the rate determined for MSA. The high rates of formaldehyde and formate oxidation may be explained, in part, by the ease of uptake of these easily diffusible molecules. Both strains were similar with respect to the oxidation of non-substituted alkyl sulfonates. There was a decrease in oxidation rate with increase of carbon chain length, to a point where no oxidation occurred for chain length of 5 carbons or more. Both marine strains showed significant differences in the oxidation rates of substituted alkylsulfonates, strain TR3 oxidized all three substituted sulfonates tested, whereas strain PSCH4 was unable to oxidize any. These trends are in agreement with those reported for the terrestrial strain M2 (Kelly et al., 1994, data reproduced in Table 5.2.).

Substrate (1.7 mM)	n	Strain TR3	Strain PSCH4	Strain M2
MSA	1	25.0	20.0	28.4
Methanol	1	9.0	6.0	32.4
Formaldehyde	1	45.8	15.0	n/r
Formate	1	33.0	11.0	4.0
Ethanesulfonate	2	10.3	10.4	10.2
Propanesulfonate	3	6.3	7.0	2.3
Butanesulfonate	4	6.0	0	0
Pentanesulfonate	5	0	0	0
Taurine	2	7.5	0	0
Isethionate	2	6.5	0	3.1
Cysteate	3	7.3	0	0
Methane	1	0	0	0
Dimethyl sulfide	2	0	0	0

**Table 5.2. Substrate specificity in whole cell experiments with MSA-grown TR3, PSCH4 and M2 (O<sub>2</sub> uptake rates).** M2 data derived from Kelly et al. (1994).

Rates expressed in nmol oxygen consumed min<sup>-1</sup>. mg dry wt cells.

All experiments carried out using MSA-grown cells. n = number of carbons. n/r; not reported.

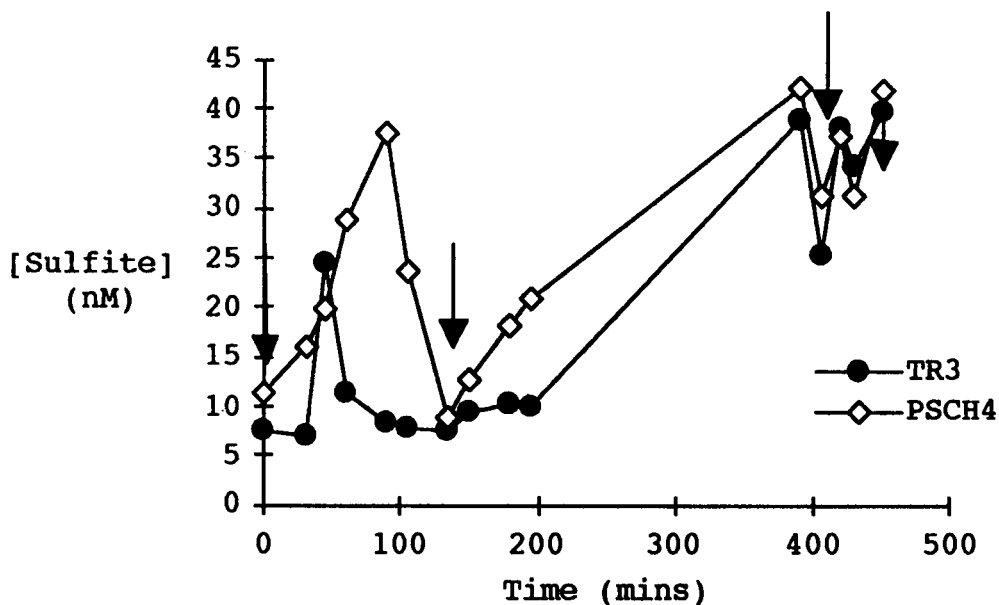
It was found that addition of 10 mM cyanide to whole cell suspensions of strains TR3 and PSCH4 did not inhibit MSA oxidation when measured using the oxygen electrode. This was considered an erroneous result, given that the MSMO reported by Higgins et al. (1995) from strain M2 showed that in cell-free extract, MSA oxidation was inhibited by much lower concentrations of cyanide. Evidence presented in this chapter and chapter 6 will suggest that MSA-specific enzyme

systems in strains TR3 and PSCH4 are probably closely related to strain M2.

### **5.2.3. Sulfite production by whole cell suspensions of strains TR3 and PSCH4.**

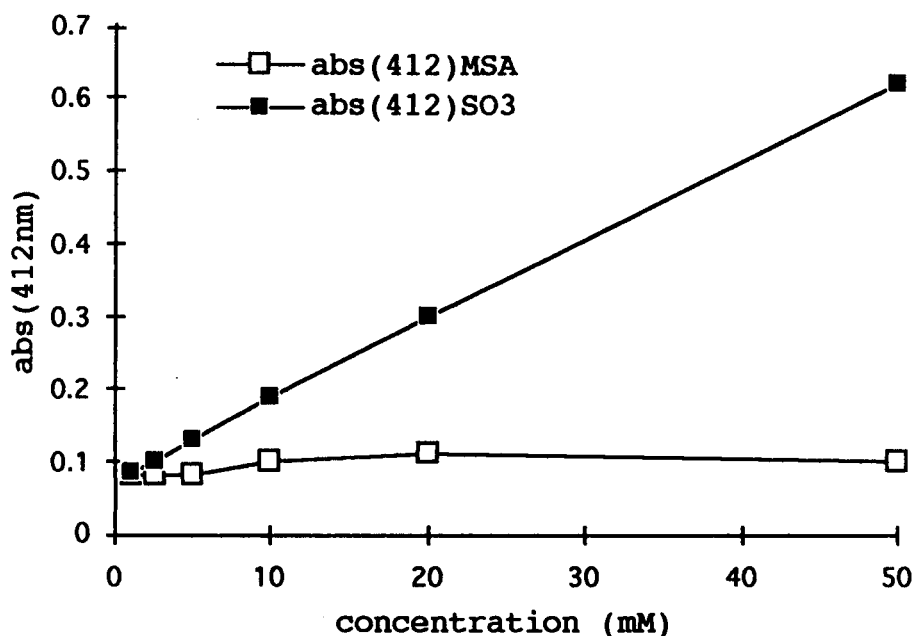
The production of sulfite by whole cells in MAMS, with MSA as a carbon source, was monitored over time using Ellman's Reagent as previously described (Figure 5.1.), and rates of between 3 to 19 nmol  $\text{SO}_3^-$  produced  $\text{min}^{-1}$  mg dry wt cells $^{-1}$  in strain TR3, and 6 to 14 nmol  $\text{SO}_3^-$  produced  $\text{min}^{-1}$  mg dry wt cells $^{-1}$  in strain PSCH4 were observed.

Sulfite production was clearly induced by the addition of MSA, indicating that this was a primary oxidation product in both strains oxidizing MSA rather than sulfate. No sulfite was produced using methanol as a carbon source indicating that sulfite production was not an artefact of general metabolism (derived from the medium). Sulfite was also produced in cell-free protein extracts of both strains (from cells grown on MSA), after the addition of MSA.



**Figure 5.1. MSA-stimulated sulfite production by strains TR3 and PSCH4, measured using Ellman's Reagent.** 50 ml MSA-grown cultures of strains TR3 and PSCH4 were incubated at 30°C and MSA was added in 100  $\mu$ mol aliquots (initial concentration of 2mM) at the start of and during the experiment (at 0, 145, and 405 minutes, indicated by the arrows). Sulfite was measured using Ellman's Reagent, described in Methods and Materials. Strains TR3 and PSCH4 were resuspended in buffer to a final concentration of 0.90 mg dry wt. ml<sup>-1</sup> and 0.40 mg dry wt. ml<sup>-1</sup> respectively.

A simple experiment, using solutions of MSA and sulfite, showed that MSA did not cross-react with Ellman's Reagent (Fig. 5.2.).



**Fig. 5.2. Experiment to show the reactivity of MSA with Ellman's Reagent.** Solutions of MSA and sulfite in double-distilled water (0, 1, 2.5, 5.0, 10, 20, 50 mM) were mixed with Ellmans Reagent as described in section 2.1.6.2., and the absorbance measured at 412 nm.

### **5.3. BIOCHEMICAL CHARACTERISATION OF ISOLATES TR3 AND PSCH4.**

#### **5.3.1. Oxidation of MSA in cell-free extracts of strains TR3 and PSCH4.**

In cell-free extracts of strains TR3 and PSCH4, an MSA specific oxygenase was detected using a modified form of the assay described by Kelly *et al.* (1994), and described in full in section 2.8.4. The apparent  $K_m$  and  $V_{max}$  were determined for this enzyme in both strains (Table 5.3.).

Enzyme	Strain TR3	Strain PSCH4	Strain M2
MSA Monooxygenase <sup>a</sup> $K_m$	0.64 mM	0.10 mM	0.02 mM
$V_{max}$	11.40	12.50	229.00
Methanol $K_m$	0.33 mM	0.14 mM	0.24 $\mu$ M
Dehydrogenase <sup>b</sup> $V_{max}$	38.90	11.00	115.00
Hydroxypyruvate $K_m$	0.22 mM	0.13 mM	4.4 $\mu$ M
Reductase <sup>a</sup> $V_{max}$	130.30	316.00	1120.00
Serine Glyoxylate $K_m$	0.89 mM	2.70 mM	n/r
Aminotransferase <sup>a</sup> $V_{max}$	93.30	182.00	n/r
Formate $K_m$	0.67 $\mu$ M	0.46 $\mu$ M	3.53 $\mu$ M
Dehydrogenase <sup>c</sup> $V_{max}$	41.80	69.00	47.00

**Table 5.3. Activities of various C<sub>1</sub> enzymes in cell-free extracts of strains TR3 and PSCH4. M2 data for comparison from Kelly et al. (1994). <sup>a</sup>  $V_{max}$  values expressed in nmol NADH min<sup>-1</sup>mg<sup>-1</sup>. <sup>b</sup>  $V_{max}$  values expressed in nmol DCPIP min<sup>-1</sup>mg<sup>-1</sup>. <sup>c</sup>  $V_{max}$  values expressed in nmol NAD<sup>+</sup> min<sup>-1</sup>mg<sup>-1</sup>. Values determined by Lineweaver-Burk and Eadie-Hofstee Plots, linear regression coefficients were in the range 0.95-0.98.  $K_m$  values are apparent values. n/r; not recorded.**

For both strains the enzyme did not use NADPH as an *in-vitro* electron donor (assays were normally carried out using NADH as an electron donor), the NADH-linked assay was characterised by a very high endogenous rate (typically, 50% of the total). MSA oxidation above the background rate could not be detected in extracts prepared from methanol-grown cells. Using a Tris

buffer containing 3.0%(w/v) NaCl did not significantly affect the activity of the oxygenase in either strain.

As described in section 5.2., oxidation of MSA in cell-free extracts was also demonstrated spectrophotometrically by using Ellman's Reagent to detect sulfite production. It was not possible to accurately determine rates using this method, since there was a background level of sulfite and thiols in cell-free extracts which cross-reacted with Ellmans reagent. This effect was studied in some detail by Johnston et al. (1975). However, the high extinction coefficient of the product ( $\epsilon = 13\ 100$  at 412 nm) ensured that the assay was sensitive enough to demonstrate the production of sulfite. In some extracts it was difficult to show activity by the NADH-linked method, but activity could be shown with the sulfite-linked assay. The MSA oxygenase appeared to be relatively unstable in cell-free extracts, with the activity decreasing even with the (thawed) extract stored on ice (typically activity halved after about 10 minutes in both strains). In order to achieve consistent activities it was necessary to use only small volumes of cell-free extract from  $-70^{\circ}\text{C}$  stocks. Typically, 200  $\mu\text{l}$  of extract was thawed, which was sufficient for 3 to 4 assays. Ultra-centrifugation of crude cell-free extracts (100,000  $g$ , 4 hours,  $2^{\circ}\text{C}$ ) did not affect this instability, nor was there a decrease in the high NADH-linked endogenous rate observed.

### 5.3.2. Oxidation of methanol in cell-free protein extracts.

Both strains possessed an active PQQ-linked MDH in cell-free extracts prepared from MSA-grown cells (Table 5.3.). When using high amounts of protein (i.e. more than 1.25 mg) in an assay, a very high "endogenous" rate was observed in both strains in the absence of added methanol. This endogenous rate was not constant and not reproducible. Also, a precipitation reaction occurred upon addition of phenazine ethosulfate (PES) in some extracts (prepared as previously described). Hence, accurate determinations of biochemical parameters ( $K_m$ ,  $V_{max}$ ) were difficult to obtain. It was necessary to use small quantities of cell free extract (0.2 mg typically) to reduce this "endogenous" rate to acceptable levels. This effect has been attributed by Anthony (1993) to an unidentified substrate already bound to the enzyme. The precipitate reaction has not been documented, but may be due to a pH change within the cuvette leading to a precipitation of proteinaceous material.

A postgraduate student, E. Kenna, successfully obtained a PCR product corresponding to *mx<sub>a</sub>F* (previously known as *moxF*, see Goodwin and Anthony, 1995), using *mx<sub>a</sub>F*-specific primers, confirming the presence of MDH within strain TR3. The gene, *mx<sub>a</sub>F*, encodes for the  $\alpha$ -subunit of MDH and is found in many

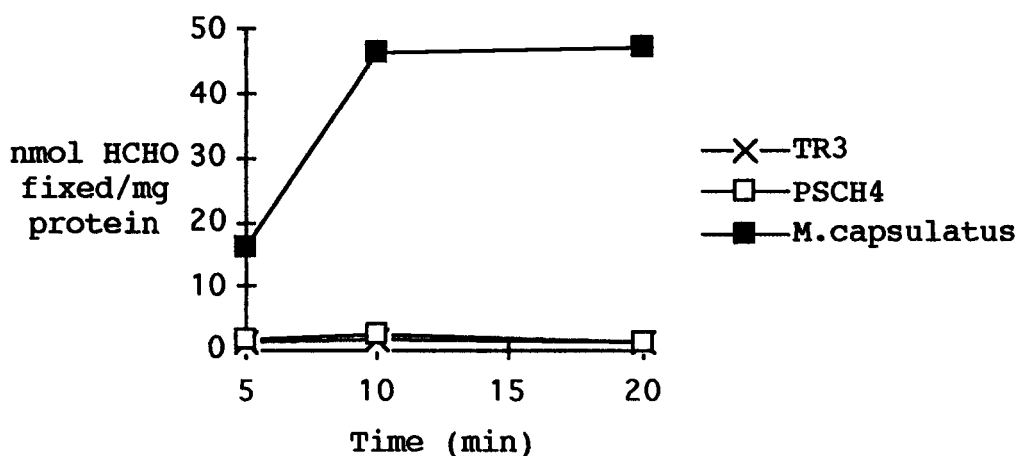


methylo trophs (Goodwin and Anthony, 1995). Chromosomal DNA from strain TR3 was prepared by the method described in 2.10.1. PCR was carried out as described by McDonald et al. (1995).

### 5.3.3. Assimilation of formaldehyde in cell-free protein extracts.

Hydroxypyruvate reductase (HPR) and serine glyoxylate aminotransferase (SGA) were both detected in cell-free extracts of strains TR3 and PSCH4 (Table 5.3.).

Hexulose phosphate synthase (HPS) was not detected in similar extracts (Fig. 5.3.) from either strain.



**Figure 5.3. Hexulose phosphate synthase activity in cell-free extracts of TR3, PSCH4 and *Methylococcus capsulatus* (Bath).** Activities determined by  $\text{H}^{14}\text{CHO}$  (specific activity =  $65\,000\text{ cpm }\mu\text{mol}^{-1}$ ) fixation in cell-free extracts of strains TR3, PSCH4 and *M. capsulatus*. Amount of formaldehyde fixed per mg of cell-free extract is an average of 3 separate determinations.

The presence of HPR and SGA and absence of HPS in cell-free extract indicates that both strains are Serine cycle facultative methylotrophs, similar to strain M2 (Kelly et al., 1994). Preliminary studies on the two freshwater strains FW2 and FW6 also indicated the presence of HPR (specific activities (in  $\text{nMol min}^{-1} \text{mg}^{-1}$ ): FW2, 335; FW6, 102).

The activities found for HPR in both strains are somewhat lower than those found for strain M2 (Kelly et al., 1994), in particular the  $K_m$  values are much higher. The reason for this is not clear. Experimental error was unlikely since extracts prepared from both strains, at different times and assayed on different occasions, gave similar results (in terms of orders of magnitude) and linear regression coefficients obtained for both were excellent, being in the range 0.95-0.98. A possible explanation was that the buffers used were prepared in non-saline Tris buffer (see chapter 2). It is not known if the internal salinity of the cell approaches that of the environment, or whether there is some sort of osmoregulator. In either case, diluting protein extracts in a buffer of potentially different pH would certainly affect the efficiencies of enzymes. The assay for MSMO was carried out on extracts of TR3 diluted in saline Tris buffer (3.0% (w/v) NaCl) and extracts diluted in non-saline Tris buffer. There was little difference in the rates recorded at the same concentration of MSA. It was not possible to carry out the MDH assay using buffer

containing 3.0% (w/v) NaCl due to the precipitation reaction previously discussed. The MDH assay is normally carried out at a high pH, and it was thought that the addition of salt may alter the pH, causing the precipitation (P.Wilkins, personal communication). For convenience, a decision was made to standardise the buffer used in diluting the protein extracts to a non-saline Tris buffer. Due to the apparent instability of the MSMO in crude extracts, dialysis of the saline cell extract would have lead to unacceptable reduction in activity.

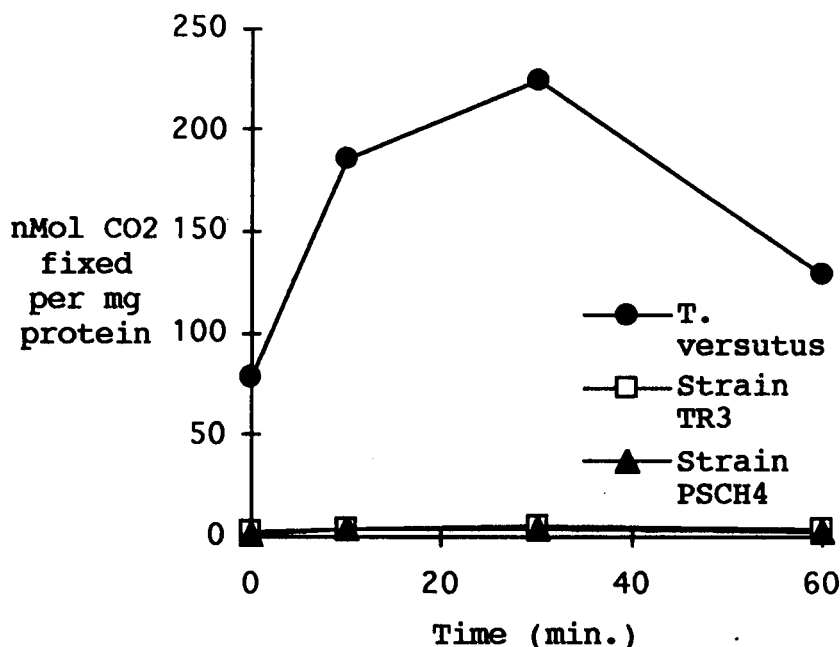
#### **5.3.4. Oxidation of formate in cell-free protein extracts of strains TR3 and PSCH4.**

Formate dehydrogenase was detected in cell-free extract prepared from MSA-grown cells of both strains (Table 5.3.), confirming that formate was completely oxidized to carbon dioxide.

#### **5.3.5. Carbon dioxide fixation by RuBISCO in cell-free protein extracts of strains TR3 and PSCH4.**

RuBISCO was not detected in either strain using the *in-vitro* biochemical assay, although it was detected in the control cell-free extract (*Thiobacillus versutus*) prepared at the same time in a similar fashion (Fig. 5.4.). Although this would seem to indicate that RuBISCO was not present in either marine strain, one cannot discount the possibility that

RuBISCO in these strains is unstable, and therefore undetectable by conventional means.



**Figure 5.4. RuBISCO activity in strains TR3, PSCH4 and *Thiobacillus versutus*.** Reactions were carried out using  $^{14}\text{C}$ -labelled bicarbonate and  $0.9\ \mu\text{mol}$  of ribulose-1,5-bisphosphate. Specific activity of radioactive reaction mixture was  $6.01\ \mu\text{Ci ml}^{-1}$ . In the case of *T. versutus*, the reaction was assumed to be complete after 30 min, to enable the  $[\text{CO}_2]$  fixed to be calculated. Data are an average of 3 separate determinations.

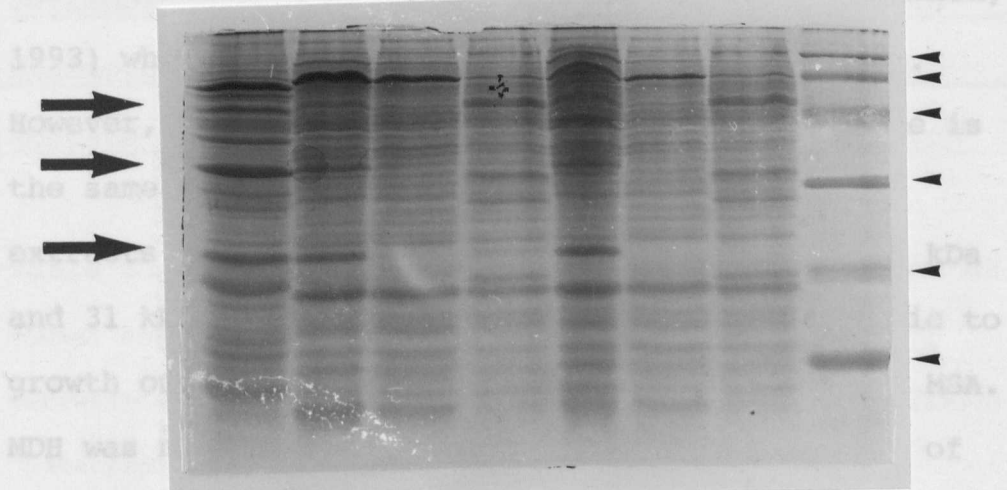
The whole cell assay was not attempted since it may have given false and misleading results. Previous biochemical studies with the terrestrial strain M2 indicated that it may possess RuBISCO (Baker, 1992). Subsequent assays (both biochemical and immunological) indicated that RuBISCO was not present (Kelly et al., 1994). The presence of the extracellular material

found in the marine strains was not conducive to the necessary cell-wall permeabilisation step required for the whole-cell assay.

#### **5.4. SDS-PAGE ANALYSIS OF CELL-FREE EXTRACTS OF MARINE STRAINS TR3 AND PSCH4.**

Figure 5.5. shows an SDS polyacrylamide gel of cell-free extracts from strains TR3 and PSCH4, when grown on a variety of substrates (MSA, methanol, monomethylamine), compared to a cell-free extract prepared from MSA-grown M2 cells. There are quite clearly a number of significant differences between MSA-grown cells and non-MSA-grown cells. Similar polypeptides were visible of (approximately) sizes 35 kDa and 25 kDa only when grown on MSA and a 40-45 kDa polypeptide was visible in both MSA and monomethylamine grown cells of both strains, though this band is difficult to size. There is no evidence of the other polypeptides appearing in extracts from MMA-grown cells. All three polypeptides were of similar size to the bands observed with strain M2 (Higgins and Murrell, unpublished results).

At around 67 kDa, a polypeptide corresponding to the large  $\alpha$ -subunit of NDH was observed for both strains grown on MSA, methanol and MMA. In MMA-grown cells, however, the polypeptide was of much lower intensity in both strains than for the other substrates. A polypeptide was seen at 43 kDa, possibly corresponding to the  $\alpha$ -subunit of NDH (Jensen, 1993) which was also observed in the other lanes.



MMA, so it is not surprising that it is only (apparently) poorly expressed. It is worth noting that NDH was highly expressed in strains TR3, PSCH4 and M2, NDH (in both marine strains) appeared to have a slightly higher molecular mass than that found in M2 (also shown in Fig. 5.5.). This probably reflected

natural variations in the subunit of NDH, and further

**Fig 5.5. SDS-polyacrylamide gel of cell extracts from strains TR3, PSCH4 and M2.** Lanes: 1; Strain M2 grown on MSA, 2; strain TR3 grown on MSA, 3; strain TR3 grown on methanol, 4; strain TR3 grown on MMA, 5; strain PSCH4 grown on MSA, 6; strain PSCH4 grown on methanol, 7; strain PSCH4 grown on MMA, 8; molecular mass markers (97, 67, 43, 30, 20.1, 14.4 kDa). The arrows indicate polypeptides specific to growth on MSA, as discussed in the text.

The relative growth rates and specific growth rates on a range of methylophilic substrates (MSA, methanol, monomethylamine) were determined for strains TR3 and

At around 67 kDa, a polypeptide corresponding to the large  $\alpha$ -subunit of MDH was observed for both strains grown on MSA, methanol and MMA. In MMA-grown cells, however, the polypeptide was of much lower intensity in both strains than for the other substrates. A peptide was seen at 45 kDa, possibly corresponding to the  $\alpha$ -subunit of methylamine dehydrogenase (Davidson, 1993) which oxidizes methylamine to formaldehyde. However, it is not clear whether this polypeptide is the same as that found in MSA-grown cell-free extracts. Several other polypeptides (20 kDa, 28 kDa and 31 kDa) were observed in both strains specific to growth on MMA, and not to growth on methanol and MSA. MDH was not directly required for the metabolism of MMA, so it is not surprising that it is only (apparently) poorly expressed. It is worth noting that MDH was highly expressed in strains TR3, PSCH4 and M2, MDH (in both marine strains) appeared to have a slightly higher molecular mass than that found in M2 (also shown in Fig. 5.5.). This probably reflected natural variations in the subunit of MDH, and further shows that strains TR3 and PSCH4 are different from the soil bacterium M2.

#### **5.5. OXIDATION OF OTHER C<sub>1</sub> SUBSTRATES IN STRAINS TR3 AND PSCH4.**

The molar growth yields and specific growth rates on a range of methylotrophic substrates (MSA, methanol, monomethylamine) were determined for strains TR3 and

PSCH4, as described in section 2.7.2., and compared to those determined for acetate (Tables 5.4. and 5.5.).

Substrate	Molar growth yield (g (dry wt) mol <sup>-1</sup> )		
	Strain TR3	Strain PSCH4	Strain M2
MSA	2.50-5.70 <sup>a</sup>	2.70-5.50 <sup>a</sup>	8.0-14.2 <sup>c</sup>
Methanol	20.34	10.65	12.6 <sup>c</sup>
Monomethylamine	5.00	3.80	4.9 <sup>c</sup>
Acetate	8.70	10.01	25.9 <sup>b</sup>

**Table 5.4. Growth yields of strains TR3, PSCH4 and M2.**

<sup>a</sup>Lower and higher values were calculated for flask and fermenter-grown cultures respectively. <sup>b</sup>Data from Baker (1992).

<sup>c</sup>Data from Kelly et al. (1994)

For MSA, values determined for both flask-grown (non-pH controlled) and fermenter-grown (pH-controlled) cells are shown. Growth on MSA was comparable to that reported by Kelly et al. (1994) for strain M2, although growth yields are considerably lower.

Growth rates on other substrates (Table 5.5) was generally much poorer for both marine strains, compared to those determined for strain M2, although growth yields were very similar. The reason for this is not readily apparent, as the marine strains' growth media was of similar type to that used for strain M2 except for the addition of NaCl.



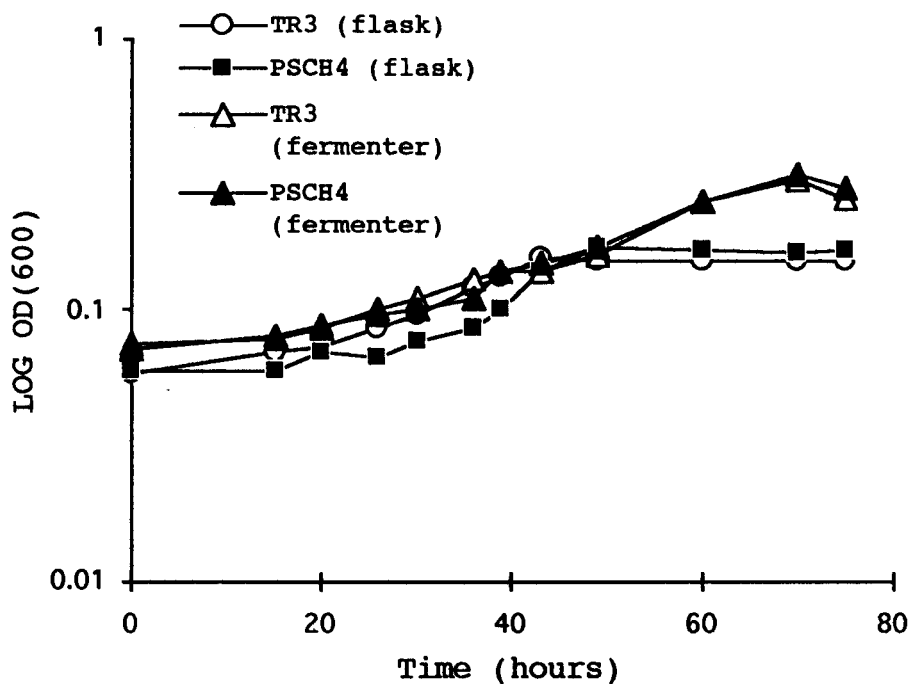
Substrate	Specific Growth Rate ( $\text{h}^{-1}$ )		
	Strain TR3	Strain PSCH4	Strain M2
MSA	0.1-0.13 <sup>a</sup>	0.10-0.13 <sup>a</sup>	0.10 <sup>c</sup>
Methanol	0.05	0.05	0.14 <sup>c</sup>
Monomethylamine	0.05	0.05	0.11 <sup>c</sup>
Acetate	0.07	0.08	0.12 <sup>b</sup>

**Table 5.5. Growth rates of strains TR3, PSCH4 and M2.**

<sup>a</sup>Lower and higher values were calculated for flask and fermenter-grown cultures respectively.<sup>b</sup>Data from Baker (1992).

<sup>c</sup>Data from Kelly et al. (1994)

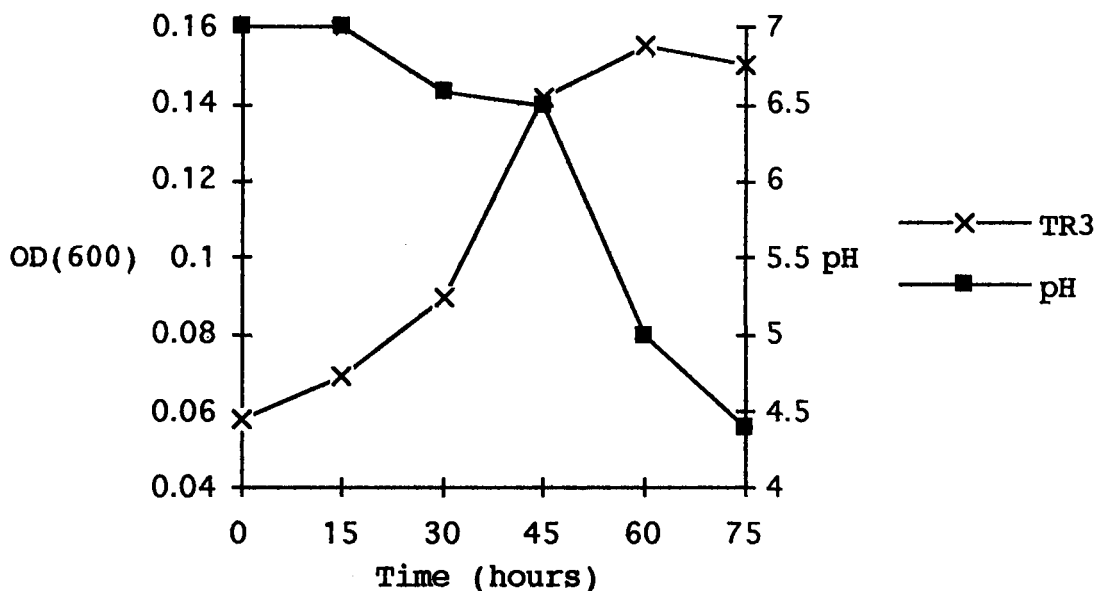
Growth yields on MSA were higher when measured in pH-controlled fermenter systems than for non-pH controlled flasks (Table 5.5). Figure 5.6. illustrates the growth curves obtained for TR3 and PSCH4 in flask and fermenter culture.



**Figure 5.6. Growth of strains TR3 and PSCH4 on MSA in flask (non-pH controlled) and fermenter (pH-controlled) culture.**

Data plotted from fermenter data sheets (typical run) vs. data from flask culture. Data plotted at same time intervals. [MSA]= 25 mM in both cases.

The molar growth yield (estimated from dry weights) obtained from fermenter experiments was on average more than twice that determined in flasks. It was shown that with the strain TR3, in a phosphate buffered medium, the pH decreased from 7.0 to 4.5 after 75 hours of incubation. This was roughly coincident with the onset of exponential growth (Fig 5.7.).



**Figure 5.7. Changes in pH during growth on MSA by strain TR3.** Data not shown on log scale. Culture grown in phosphate-buffered MAMS, with 25mM MSA, on rotary shaker at 30°C.

## 5.6. DISCUSSION.

The MSA catabolic pathway indicated by the whole cell oxidation data was confirmed by the subsequent cell-free extract experiments. The MSA-specific mono-oxygenase assay showed the presence of a NADH-linked MSA mono-oxygenase (MSMO) in both marine strains. This enzyme is apparently similar to that observed in the terrestrial bacterium, strain M2 (Kelly et al., 1994). The oxygen: substrate stoichiometry data also suggests that the initial step of MSA oxidation is a mono-oxygenation step, resulting in desulfonation of MSA and producing formaldehyde. MSA oxidation could not be shown in non-MSA grown cells (or extracts prepared

from MSA-grown cells). If the "specific" polypeptides observed in extracts of MSA-grown cells by SDS-PAGE are the subunits of a monooxygenase, then this suggests that the monooxygenase is substrate inducible. The number of these "specific" polypeptides suggests the presence of a monooxygenase of at least 2 subunits, similar to that observed in strain M2 (Higgins et al, 1995). Although no purification of the enzymes from either marine strains was attempted it is possible that both strains would possess a structurally similar enzyme system to strain M2, since the "specific" peptides are of similar size to those found for strain M2. Some degree of instability of the enzyme in cell-free extracts has been observed in strain M2 (Higgins, personal communication), though not to the same degree as observed with both marine strains. The difference in stability of MSMO between the marine and terrestrial strains is possibly a function of the buffer used in the assay, and so may indicate a difference in the pI of the enzyme in the marine strains compared to strain M2. Other enzymes assayed (HPR, SGA, MDH, FDH) showed similar stability to that observed in strain M2.

Methanol oxidation was detected in MSA-grown cells of both marine strains, using both the whole-cell (oxygen electrode) and cell-free extract (DCPIP-linked assay) assays. Although MDH was detected in cell-free extracts of MSA-grown cells, and oxidation of methanol could be demonstrated in these MSA-grown cells, this

does not necessarily imply a direct role in the oxidation of methanol. The use of PQQ-specific inhibitors (cyclopropanol) did not inhibit MSA oxidation, nor was methanol detected in culture supernatant. The high level of endogenous activity observed in cell-free extracts of these cells indicates that MDH synthesis within these cells was induced by the presence of a suitable substrate. Anthony (1993) has suggested that the endogenous activity is most probably due to the presence of MDH-bound substrate in these extracts. Stouthammer (1992) has suggested MDH may play a role in cell detoxification by removing excess formaldehyde (MDH can be shown to oxidize formaldehyde), this may be occurring here in the case of strains TR3 and PSCH4. There is no evidence that such a detoxification mechanism, if it exists, is coupled to energy conservation. The reason why MDH is expressed is clearly something to be resolved, for it has not been fully explained in isolate M2 (Baker, 1992).

Both strains were able to utilise formaldehyde in whole cell assays and cell-free extract experiments. From the results illustrated in chapter 4, it is not surprising that formaldehyde was not a suitable growth substrate for strain PSCH4, since it is toxic to many cells. More unusually, strain TR3 was able to grow with formaldehyde as the sole carbon source (at 12.5 mM). TR3 and PSCH4 both possess the Serine pathway enzymes for the assimilation of formaldehyde; high

activities of both hydroxypyruvate reductase and serine glyoxylate aminotransferase strongly support this notion. That neither organism possessed the RuMP pathway is not unexpected, given that other terrestrial MSA-oxidizing strains have been shown to possess the Serine cycle [e.g. strain M2 (Kelly et al., 1994), strains FW2, FW6 (from activity measurements of HPR), various uncategorised MSA oxidizing soil and freshwater bacterial isolates (Murrell, unpublished results)]. Few generalisations can be drawn from this regarding the systematics of these isolates, except to say that most gram positive facultative methylotrophs possess the RuMP pathway, whereas for gram negative bacteria both the Serine cycle and RuMP pathways are found (Green, 1992, Stouthamer, 1992).

The sulfite production experiments indicated clearly that sulfite was the primary oxidation product from MSA, rather than sulfate. This is similar to strain M2 (Kelly et al., 1994) and other bacterial species utilising sulfonates (Lee and Clark, 1993, Hansen et al., 1992, Biedlingmaier and Schmidt, 1983). Sulfate has been suggested to be the primary oxidation product in some enteric bacteria and fungi (Willems, 1973, Zurrer et al., 1987). Others have found that although sulfite is initially produced, it may be subsequently oxidised to sulfate within the cell (Uria-Nickelsen, 1993). What is clear from the whole cell experiments described is that strains TR3 and PSCH4 treated

sulfite in a similar manner, i.e. there was only a short lag phase between MSA being added to the media and sulfite being produced, suggesting that sulfite is not sequestered within the cell. The rates found compared favourably with the oxygen uptake rates found with cells spiked with MSA. If one assumes 2 molecules of oxygen is required to oxidise MSA, and that the maximum oxygen uptake rate is 20-25 nmol  $O_2$  min<sup>-1</sup> mg dry wt. cells (derived from Table 5.2.) for both marine strains, then one can estimate the MSA oxidation rate (assuming all the oxygen used is for MSA oxidation) to be between 10 to 12 nmol MSA min<sup>-1</sup> mg dry wt. cells<sup>-1</sup>. The sulfite production rates were very similar to this estimate (between 3 to 19 nmol  $SO_3^-$  produced min<sup>-1</sup> mg dry wt. cells for strain TR3 and 6 to 14 nmol  $SO_3^-$  produced min<sup>-1</sup> mg dry wt. cells). If one assumes a 1:1 relationship between MSA consumed and sulfite produced, this implies that little, if any, MSA-derived sulfur was retained within the cell. The disparities between oxygen-electrode derived results and results from Ellman's reagent experiments can be explained by the differences in the sensitivities of the two techniques. With the oxygen electrode experiments oxygen was measured directly, whereas for sulfite it was the production of the Ellman's-sulfite complex that was measured and not sulfite directly, introducing a degree of error. Significant error may occur in assumptions made in deriving MSA-oxidation rates.

If sulfite was the primary oxidation product (in terms of being produced early on in the oxidation of MSA), it is not surprising that the molar growth yields and growth rates were depressed in flask-grown batch cultures, when compared with fermenter-grown batch cultures. In aqueous solution sulfite would be rapidly oxidized to sulfate, which is then hydrolysed to sulfuric acid. The phosphate buffer used in the medium was relatively weak, indeed experiments with strain TR3 showed that the buffering used was inadequate during a normal length growth experiment (i.e. 60 hours). With strain TR3, a drop in pH to 4.5 was concomitant with an onset of a stationary phase of growth indicating that batch cultures were pH-limited, not substrate-limited. Given the close similarity between strains TR3 and PSCH4, it would be reasonable to expect a similar pattern of pH change with strain PSCH4. However, changes in pH had only a relatively small effect in the specific growth rate of strain TR3, although this is probably an artefact of the buffering system used. The large discrepancy between yields obtained for strain M2 (3 to 4 times larger) may be a result of some missing growth factor in MAMS, required by both marine strains. The growth yields determined for methanol and monomethylamine are lower than those predicted by Anthony (1978) (Table 5.6.), but are within the range predicted for serine-pathway methylotrophic bacteria possessing isocitrate lyase ( $icl^+$ ) and NADH.



Substrate	g dry wt per mole substrate			
	RuMP	Serine Pathway (icl <sup>+</sup> ) <sup>a</sup>	Serine Pathway (icl <sup>+</sup> ) <sup>b</sup>	Serine Pathway (icl <sup>-</sup> )
Methanol	22.6	14.2	21.4	12
Monomethylamine	22.6	14.2	21.4	12

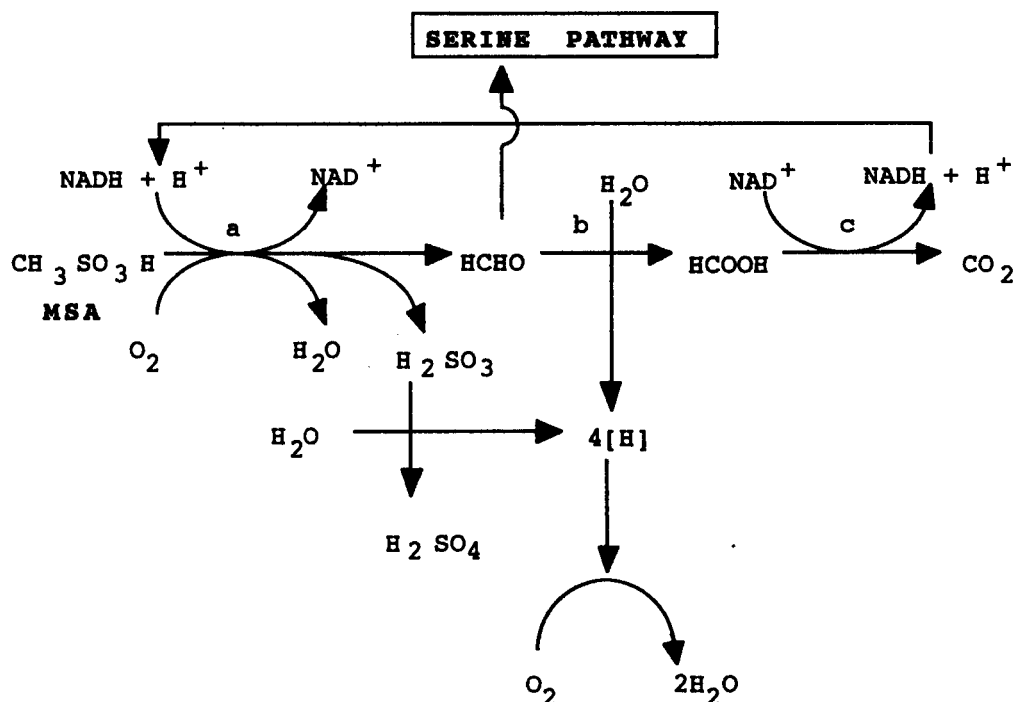
**Table 5.6. Predicted molar growth yields (from Anthony, 1978). icl; isocitrate lyase. <sup>a</sup>Reducing equivalents not recycled. <sup>b</sup>Reducing equivalents (NADH) recycled.**

Both values are similar to that reported by Kelly et al. (1994) for strain M2, and so may represent a trait amongst MSA-oxidizing bacteria.

## 5.7. CONCLUSIONS.

The evidence presented indicates the methanogenic route discussed does not occur in either strain. The sulfonate moiety is not cleaved off as sulfate but as sulfite, as indicated by whole-cell and cell-free assays. Methane was not a growth substrate for either strain, nor was there any evidence of RuBISCO being present. This is in agreement with that found in the strain M2 (Kelly et al., 1994). The evidence does indicate the existence of a NADH-dependant inducible monooxygenase system oxidizing MSA directly to formaldehyde, which is then fed into the Serine cycle (for assimilation into cell biomass) or is mineralised to carbon dioxide via formaldehyde dehydrogenase and formate dehydrogenase. The evidence suggests that

methanol is probably not directly produced from the oxidation of MSA. These observations are summarised in the proposed reaction scheme (Fig. 5.8.).



**Figure 5.8. Proposed pathway for the oxidation of MSA in marine strains TR3 and PSCH4.** <sup>a</sup>MSA-inducible monooxygenase, <sup>b</sup>formaldehyde dehydrogenase (not assayed for), <sup>c</sup>formate dehydrogenase.

The MSA-specific monooxygenase has a relatively narrow substrate specificity, confined to alkylsulfonates of less than 3 carbons, and shows the highest specificity for MSA.

Methanol dehydrogenase was expressed during growth on MSA, but its function is unclear, it may be involved in cell detoxification.

## CHAPTER 6: CONCLUDING REMARKS.

### 6.1. MSA OXIDATION BY STRAINS TR3 AND PSCH4.

#### 6.1.1. Biochemical aspects.

Two MSA-oxidizing methylotrophic bacterial strains, TR3 and PSCH4, were isolated from seawater, after a comprehensive enrichment programme. Strains TR3 and PSCH4 both utilised MSA as a sole source of carbon and energy, with the concomitant release of sulfite. There is no evidence to suggest that the sulfite is further oxidized within the cell to sulfate, as has been observed in other bacteria (Uria-Nickelson *et al.*, 1993a, b). From an ecological viewpoint, this is to be expected, since where MSA is found, sulfate is also likely to be found (the same series of atmospheric reactions that degrade DMS to MSA also produce sulfate). Hence there is unlikely to be a need for the bacterium in question to utilise the sulfur from MSA *per se*. MSA in both strains was initially oxidized to formaldehyde by a specific NADH-dependent monooxygenase. Studies of the two marine strains, TR3 and PSCH4, and M2 and other freshwater/terrestrial MSA oxidizers, have shown a high level of similarity, in terms of action and apparent structure of the monooxygenase, such that it would seem reasonable to call the monooxygenases in all these strains methanesulfonic acid monooxygenase (MSMO).

Studies on MSMO from strain M2 are at an early stage, with the main components still to be fully characterised. However, clearly this is a multicomponent monooxygenase of the type described as alkyl-group hydroxylases by Harayama et al. (1992). The following components have been identified (Higgins et al., 1995, in press); component A, a reductase (36 kDa), component B, a heterotetramer hydroxylase of 200000 kDa and component C, a 16 kDa component identified as a ferridoxin protein. A 45 kDa MSA-specific polypeptide has been purified and sequenced, although it is of unknown function.

It seems likely that MSA is only present at a low concentration in seawater (in the nM range). The relatively high  $K_m$  values for MSA oxidation reported here are significantly higher than MSA seawater concentrations. Unfortunately, in this present study, it was not possible to determine the  $k_m$  of MSMO in whole cells satisfactorily by measuring MSA oxidation directly. The oxygen electrode was not sensitive enough to detect oxygen consumption at very low MSA concentrations (i.e. low nM range). Ellman's Reagent, although very sensitive for detecting low concentrations of sulfite, could exert inhibitory effects on MSA oxidation (e.g. from toxic effects) which might only be apparent at very low oxidation rates (i.e. that might be expected when oxidizing MSA metabolism at very low concentrations). For studies of

MSA oxidation at low concentrations in cell suspensions, the Dionex chromatography system would be ideal, due to its high sensitivity to very low concentrations of MSA and that its use would not affect MSA oxidation in cells (unlike Ellman's Reagent).

#### **6.1.2. Molecular characterisation of MSA oxidizing isolates.**

The phylogenetic position of strains TR3 and PSCH4 is uncertain. The phylogenetic position of strain M2 has recently been ascertained by sequencing the 16S rRNA gene (A.J. Holmes, personal communication). An unrooted 16S rRNA tree is presented in Appendix I. M2 falls into the  $\alpha$ -proteobacteria group but is not closely related to *Methylobacterium extorquens* and *Methylocystis parvus*. Further clarification is required, but M2 may represent a new methylotrophic species. Physiological and biochemical studies presented here suggest a high degree of relatedness between strains TR3 and PSCH4, in the first instance, and between both marine strains and strain M2. Time constraints did not allow the 16S rRNA gene of strains TR3 and PSCH4 to be fully sequenced during the course of this study. However, other workers at the University of Warwick are currently sequencing the 16S rRNA gene in strains TR3, PSCH4 and other isolates. Initial probing experiments, using an M2-specific 16S rRNA gene signature sequence, showed strain M2 and

other uncharacterized MSA-oxidizing bacteria, and marine strains (strains TR3 and PSCH4) falling into two distinct groups (P. De Marco, personal communication). Agarose gel electrophoresis banding patterns (produced by treating the probed DNA with *XhoI*) showed that the soil and freshwater strains appeared to be virtually identical to strain M2, whereas strains TR3 and PSCH4, though similar, showed significant differences to M2.

Additionally, work is being carried out by workers at the University of Warwick to identify and sequence MSMO in strain M2 at both the protein and gene level. Two subunits have been partially DNA sequenced (the genes encoding the 45 kDa polypeptide and the 16 kDa ferridoxin, component C), and oligonucleotides designed to them (P. De Marco, personal communication). A 6.5 Kb *SphI* DNA fragment was isolated from M2, containing both gene sequences and used to probe chromosomal DNA of strains TR3, PSCH4, M2 and a variety of freshwater/ terrestrial isolates. Similarly to that found using the 16S rRNA based probe, marine and freshwater strains fell into two distinct groups, on the basis of restriction enzyme banding patterns. The freshwater strains were showed to have several *XhoI* sites, whereas the marine strains apparently only had one, leading to fragments of approximately the same size in both marine strains.

## 6.2. ENRICHMENT PROCEDURES.

All the sites sampled yielded putative MSA utilizing bacterial enrichment cultures. It was noticeable that enrichment cultures were more readily obtained from samples taken in the summer than from winter samples, even from water of the same locality. The success of future enrichments may be substantially enhanced by confining seawater samples to those obtained in the summer or spring. As noted in chapter 1, the incidence of culturable bacteria seems to decrease with the onset of winter (perhaps due to the decrease in primary productivity).

The enrichment experiments using the tangential flow filtration also showed that either the marine methylotrophic bacteria are substantially smaller than might be supposed from observations of laboratory cultures (typically 0.5-1.0  $\mu\text{m}$  length or breadth), or that the efficiency of filters routinely used to obtain a representative bacteria population in most enrichments is in doubt. The use of tangential flow filtration permits the use of filters with very small cut-off sizes to efficiently screen large volumes of water far more quickly than with conventional filters. These filter systems are also convenient to use for field work sampling (e.g. at sea).

### **6.3. FINAL CONCLUSIONS: THE MICROBIAL OXIDATION OF METHANESULFONIC ACID IN THE MARINE ENVIRONMENT.**

The data presented here have not only answered many questions regarding the fate of MSA in the marine environment, but also raised new ones. It was shown that MSA-oxidizing methylotrophic bacteria are present in seawater, strengthening the hypothesis of Baker et al. (1991) that MSA utilizing bacteria are ubiquitous in the environment. The fact that presumptive MSA utilizers were found in most sites sampled suggests that MSA oxidizing bacteria are widely distributed. The metabolic status in the environment of such bacteria is unknown, and would largely depend on the availability of MSA in the environment. However, as discussed in Chapter 1, marine bacteria appear to have the ability to sequester nutrients at very low concentration, or may have survival strategies based a wide range of substrates. Both marine isolates possessed an apparently similar MSA-specific monooxygenase to the soil bacterium M2 (Kelly et al., 1994), along with other biochemical and physiological similarities. This suggests that the prospects for enumeration, using molecular techniques, are good.

### **6.4. FUTURE PROSPECTS.**

The major interest in MSA oxidation is in its role in the global sulfur cycle. A logical next step would be to enumerate MSA oxidizing bacteria in the environment, to assess their impact on the sulfur



cycle. However, there are a number of questions that need to be answered before proceeding;

#### 6.4.1. Quantity of MSA in seawater.

Although one can make estimates as to the amount of MSA deposited on land and sea from the atmosphere, this would not take any account of other mechanisms producing MSA (e.g. biological). Blom and Tangerman (1988) have already suggested that methanethiol (biologically derived from DMS in seawater) may be oxidized in the blood to formate via MSA. It seems likely that this will probably occur in other biological systems, and hence could be a possible source of MSA (and, given the prevalence of methanethiol in seawater, potentially important). Given the change of oxidation state of sulfur in methanethiol to MSA is -2 to +5, such an oxidation would probably be carried out by a chemolithoheterotrophic bacterium.

To date, there are no published data on the concentration of MSA in seawater. In Appendix II, I have briefly discussed a novel method to measure MSA in seawater using ion exchange chromatography. A considerable amount of refining is required, but the method would appear to be viable. HPLC has also been suggested as a possible method to measure MSA in seawater (R. Kiene, personal communication). Such measurements might also indicate the distribution of

MSA in the water column, and thus the likely sites of MSA-oxidizing bacteria (e.g. subsurface microlayer, see section 1.6.).

#### **6.4.2. Measurement of MSA oxidation *in-situ*.**

It is not trivial to measure MSA in seawater, nor to measure *in-situ* oxidation rates (i.e. rates measured in mesocosm-type apparatus). Chapter 1 and Appendix II outline some of the analytical problems associated with measuring MSA in seawater. It may be more feasible to measure MSA oxidation indirectly, such as by measuring sulfite (or sulfate) production. It may also be possible to measure MSA oxidation using  $^{14}\text{C}$ -labelled MSA. MSA uptake rates could be determined in mesocosms by spiking the seawater with  $^{14}\text{C}$ -labelled MSA, and determining MSA concentrations in seawater, biomass and in  $\text{CO}_2$ .

#### **6.4.3. Molecular enumeration techniques.**

Since many marine bacteria are unculturable, it would be desirable to approach the problem of enumeration using DNA probing technology. However, this assumes a certain degree of relatedness or homogeneity of the targeted bacteria, typically within the 16S rRNA gene or in functional genes (*viz* MSMO). As discussed previously, initial studies of the MSA oxidizing bacteria isolated to date suggest that they are quite closely related. If the isolates described in this

work are representative of marine MSA utilizing bacteria then the prospects for enumeration, using molecular techniques, are good. The evidence presented in this work suggests that all MSA-oxidizing methylotrophic organisms fall into two closely related groups, consisting of the marine bacteria and terrestrial/ freshwater bacteria. Typical approaches that could be taken to enumerate these types of bacteria (*viz.* strains TR3, PSCH4) would involve DNA probes based upon functional genes (e.g. based on specific enzyme systems) or phylogenetic probes (e.g. 16S rRNA gene).

In general terms, PCR primers may be designed, based on the gene probe sequences (derived from functional and phylogenetic genes), and used to amplify targeted sequences within a sample. The resultant PCR products may be sequenced and compared, thus providing a measure of diversity of these MSA utilizing strains. This approach would require very specific probes, so that spurious sequences are not obtained. An alternative approach, for the phylogenetic probes, is to amplify all the 16S rRNA gene sequences within a sample, using so-called universal eubacterial primers, and probing these products with the specific phylogenetic probe. Quantitative PCR could be applied here to determine which proportion of the total bacterial population are possible MSA oxidizers. It should be noted, however, that phylogenetic probes would not necessarily determine function.

Extraction of DNA from environmental samples and subsequent PCR can present a number of technical challenges. The low amount of DNA in environmental samples presents handling problems, due to loss of sample and substances within the sample can affect the enzymes used in the PCR (e.g. humic acid derivatives), and so procedures are required to "clean-up" the sample (McDonald et al., 1995). This could obviously affect quantification of subsequent PCR signals (i.e. by loss of some amount of DNA during the preparative procedures). *In-situ* DNA hybridization technologies offer the potential for direct quantification of selected bacteria within a sample (Leisack and Stackebrandt, 1992). Fluor-tagged DNA probes of the types outlined above can be introduced into suitably treated cells (i.e. formaldehyde fixative step), and quantification made by epifluorescence microscopy (e.g. Amann et al., 1992). Multiple probes can be applied to allow the enumeration of very specific populations. Although this approach has been used for some time in medical microbiology, it has only recently been applied to environmental microbiology (e.g. to enumerate marine methane oxidizing bacteria, Holmes et al., 1995, In Press). A method such as this could be affected by either the efficiency of the cell-fixing step, or by the low numbers of target bacteria in a typical volume of seawater (although the size of the overall bacterial population can be quite significant). Use of filtration systems, such as the

tangential flow filtration system described in this work, can be used to enhance the numbers of target bacteria in a sample, to allow statistically acceptable counts to be made. Alternatively, flow cytometry could be used to sort and quantify fluorescing cells in a large volume sample (the principles behind this are outlined by Haynes (1988)).

#### **6.4.4. Determination of bacterial MSA oxidation in seawater.**

Given that MSA can be measured in seawater, and that specific gene probes will become available, it is possible to envisage an enumeration programme to determine the importance of MSA-oxidizing bacteria in the environment. At least three gene probes can be envisaged; an MSMO-specific gene probe, 16S rRNA gene region specific probe, and a *mxoF* probe (specific for  $\alpha$ -subunit of MDH, and so useful in detecting methylotrophic organisms). Counts of whole cells could be made from environmental samples using DNA probes tagged with different fluors in combination with epifluorescence microscopy or flow cytometry. Using a suite of the above probes should reduce the incidence of non-specific hybridization giving false positives (*bone fide* MSA-utilizing methylotrophs would hybridise with all the specific DNA probes used). Such enumeration could be combined with *in-situ* MSA flux measurements, so allowing a proper assessment of the importance of biological MSA oxidation.

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## APPENDICES

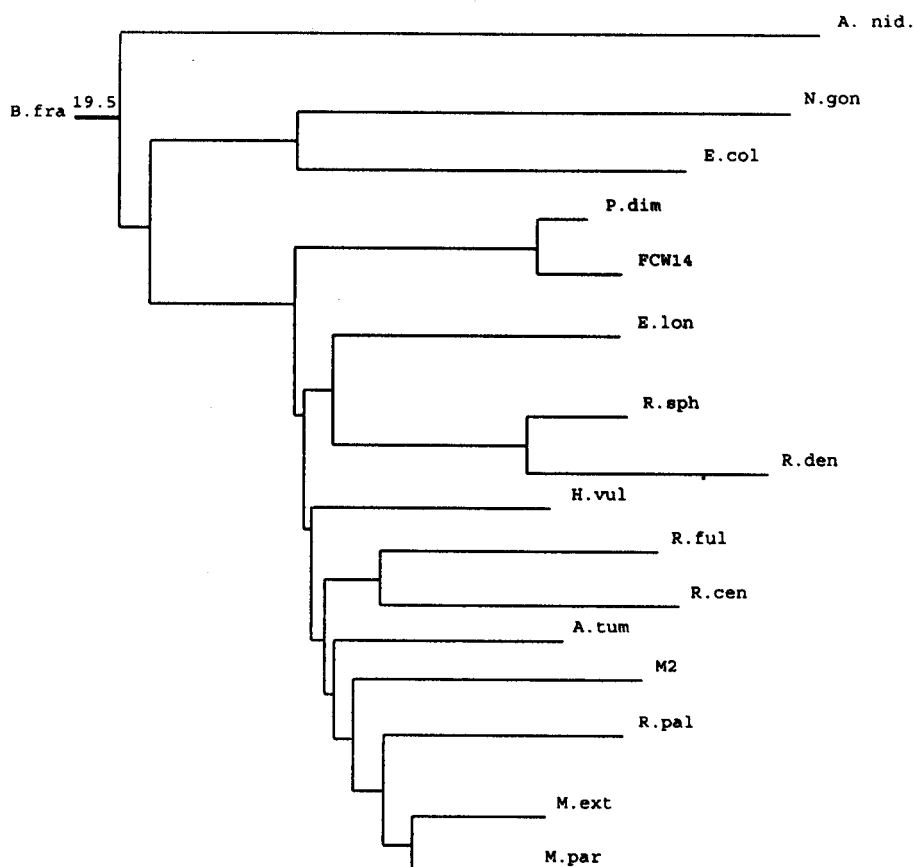
**APPENDIX I. Unrooted 16S rRNA tree showing the relationship of strain M2 to other  $\alpha$ -proteobacteria.**

Figure A1.1. shows the phylogenetic relationships of strain M2 and other proteobacteria.

**Abbreviations:**

B.fra.....	<i>Bacillus fragilus</i>
A.nid.....	<i>Anacystis nidulans</i>
N.gon.....	<i>Neisseria gonorrhoeae</i>
E.col.....	<i>Escherichia coli</i>
P.dim.....	<i>Pseudomonas diminuta</i>
FCW14.....	<i>Caulobacter</i> sp. FCW 14
E.lon.....	<i>Erthrobacter longus</i> Och 101
R.sph.....	<i>Rhodobacter sphaeroides</i>
R.den.....	<i>Roseobacter denitrificans</i> Och 114
H.vul.....	<i>Hyphomicrobium vulgare</i>
R.ful.....	<i>Rhodospirillum fulvum</i>
R.cen.....	<i>Rhodospirillum centerium</i>
A.tum.....	<i>Agrobacterium tumefaciens</i>
R.pal.....	<i>Rhodopseudomonas palustris</i>
M.ext.....	<i>Methylobacterium extorquens</i>
M.par.....	<i>Methylocytis parvus</i>

The tree was rooted with *Bacillus fragilus* as an outgroup.



— 1 cm = 1 unit of evolutionary  
distance (arbitrary value)

Fig. A1.1. Unrooted 16S rRNA tree showing the relationship of strain M2 to other  $\alpha$ -proteobacteria Taken from Holmes, Kenna and Murrell (unpublished results).

## **APPENDIX II. MEASUREMENT OF MSA IN SEAWATER BY DIONEX ION EXCHANGE CHROMATOGRAPHY.**

MSA concentrations in seawater have not been determined due to the inherent difficulties associated with using seawater in ion exchange chromatographs to detect trace salts. An attempt was made to determine MSA concentrations in seawater, with a view to using such a system to determine MSA consumption rates in seawater. Dr. S.F. Watts (Oxford-Brookes University) kindly provided technical assistance and expertise towards this end.

### **AII.1. Description of the Dionex System.**

The system used was the Dionex 4500i, utilising a high pressure (5000 psi) gradient pump, fitted with an IonPac AS4A anionic column and electronic suppresser. The eluent used was 1 mM sodium hydroxide. Detection was by a conductivity meter.

### **AII.2. Method development.**

A major problem with detecting MSA in seawater is its low concentration (1 ppb or less). Ordinarily this could not be detected satisfactorily by the Dionex system, the most sensitive IC system commercially available. The elution time of MSA, is also very close to fluoride and chloride, both present in seawater at higher concentrations. Sulfate concentration is also

very high, leading to a column overload. Several approaches were made; by concentrating seawater, using a concentrator column, and by carefully spiking seawater with MSA. In both cases, efforts were made to reduce both contaminating organic compounds and halides (principally  $\text{Cl}^-$ ). The following sample treatment was established:

- [i] Filter 10 ml sample through a 0.22  $\mu\text{m}$  filter (Whatman Nitrocellulose).
- [ii] Pass filtered sample through a Dionex Onguard-RP cartridge (removes contaminating hydrophobic molecules.
- [iii] Pass sample through 2 Dionex Onguard-Ag cartridges (removes principally chloride as silver salt).
- [iv] Load sample.

Tests using spiked seawater samples showed some MSA was removed by the RP cartridges (variable, 20-40%). MSA removal by the Ag-cartridges was small. These Ag-cartridges removed enough chloride for MSA to be detected in samples. Quantification of MSA by using spiked samples was unsatisfactory, but by using concentrated samples (10x), it was possible to detect MSA to about 0.1 ppb. The sulfate within the sample was often retained within the column, and great care was required to wash the column before further analysis. An unwelcome side-effect of using concentrated samples was that the lifespan of a column

was reduced considerably reduced, due to the high ionic strength of the solution.

### **AII.3. Conclusions.**

MSA concentrations need to be determined in seawater to satisfactorily determine the ecological role of MSA-oxidizing bacteria. A method must be developed that would successfully detect (potentially) small changes in MSA concentration. Clearly, much more development of this method described is required before it could be applied on a routine basis to environmental samples. However, this study has shown it is perfectly feasible to measure MSA in seawater by Dionex Ion Chromatography. Whether the method would be suitable for determining consumption rates involving small changes is uncertain. The sample preparation step needs to be better developed, in order to minimise losses of MSA to the filters, or to quantify this loss. It may be preferable to use  $^{14}\text{C}$  labelled MSA to determine consumption rates. Clearly, the cost of routinely measuring MSA in seawater by the Dionex system could potentially incur similar costs, due to column replacement.

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